REMARKS

Reconsideration of the present application in view of the above amendments and the following remarks is respectfully requested.

Status of the Claims

Claims 9-12, 16-18, and 23-32 were acted on by the Examiner in the Office Action dated February 10, 2006. Claims 9-12, 16-18, and 23-32 have been rejected. Claims 9-10 and 24 have been canceled. Claims 16, 25, 26, 28, 29 and 32 have been amended. Accordingly, Claims 11-12, 16-18, 23, and 25-32 are presented for examination.

Examiner Interview

The undersigned conducted an Examiner interview on March 31, 2006 to discuss the Trapnell et al. and Smith et al. publications, the Examiner's rejections, and proposed claim amendments. The Examiner indicated that adding a recitation to Claims 16 and 26 to a single administration of a vector "would be a substantial step forward" in allowing these claims. Accordingly, applicants have amended Claims 16 and 26 to add this recitation. The Examiner also indicated that *ex vivo* claims (Claim 29 and its dependents) would be more likely to advance in prosecution if applicants provided references to support the fact that *ex vivo* methods were enabled at the time of filing. Accordingly, applicants have cited and enclosed several review articles which disclose *ex vivo* protocols known in the art at the time of filing.

Section 102(a) Anticipation Rejections of Claims 26-28

Claims 26-28, directed to a method for increasing the tolerance of a mammal to transgenic cells, were rejected by the Examiner under 35 U.S.C. § 102(b) as being anticipated by the disclosure of Smith et al., *Gene Therapy* (1996); 3:496-502 (hereafter "Smith et al.") and, alternatively, by the disclosure of Trapnell et al., International Publication No. WO 96/12406 (hereafter "Trapnell et al."). The Examiner has also indicated if claim 16 is directed to the administration of a transgene rather than a transgenic product, then this rejection would apply to claim 16 and its dependents.

Smith et al. and Trapnell et al. disclose essentially the same methods. For example, compare Figure 2(b) of Smith et al. with Figure 17 of Trapnell et al. Both publications are directed to a method of administering adenovirus vectors expressing a transgene along with administration of an immunosuppressant that will decrease the formation of anti-adenovirus neturalizing antibodies to allow for a more effective second administration of the adenovirus vector. The goal of Smith et al. and Trapnell et al. is to decrease the humoral immune response to the adenoviral vectors to allow for *repeat* doses of the vector. For example, Smith et al. explicitly states that its goal is to increase the tolerance to multiple repeat doses of a vector.

> In present study we demonstrate that the humoral immune response to a systemically administered adenovirus vector is dose dependent and can be modulated by a brief treatment with the immunosuppressive agents cyclophosphamide or deosyspergualin at the time of initial treatment. This strategy permits effective *multiple repeat* doses of a vector encoding a therapeutic gene . . . . Smith et al., p. 496 (emphasis added).

Similarly, Trapnell et al. states that the object of their invention is "to provide for sustained efficacy of gene transfer via *repeated administration of adenoviral vectors*, and for sustained expression of the transferred gene, through the suppression of an immune response against the adenoviral vectors. Trapnell et al., p. 4 (emphasis added). Accordingly, Trapnell et al. and Smith et al. are focused on the transient expression of the transgenic product. This is reflected in the fact that both Trapnell et al. and Smith et al. test only for the expression of transgenic product a week after the repeat administration of the vector. Smith et al., captions to Fig. 1, 2(b); Trapnell et al. at p. 36. Trapnell et al. and Smith et al. do not test for long term expression of the transgenic product because they are primarily concerned with the humoral immune response against the viral vector to allow for repeated dosing of the vector.

In contrast, applicants' invention is directed to increasing tolerance to the transgenic cells by suppression of the cellular immune response. Specification at p. 4, lines 5-6. More particularly, the object of applicants' invention is to "prevent the rapid destruction of the transgenic cells and thus increase the tolerance of a mammal to transgenic cells. Thus, the expression of the transgenic product is maintained longer in vivo. As a consequence, "*repeated administration of the genetic material could be stopped . . . .*" Specification at p. 2,

lines 6-10 (emphasis added). In Example 2, applicants show that for at least a period of 200 days following administration of the vector, production of the transgenic product is at least 50% higher than the control. Accordingly, an embodiment of applicants' invention is a method of increasing the tolerance to transgenic cells and a method for increasing expression of a transgenic product using a *single* administration of a vector carrying a transgene.

Claims 16 and 26 have been amended to recite that the transgenic cells are produced *in vivo* after a *single* administration of a vector. Support for this amendment can be found on p. 2 and Examples 1 and 2. Claims 11-12, 17-18, 23, and 25 depend from Claim 16. Claims 27-28 depend from Claim 26. Claim 16 has also been amended to make clear that the method is directed to the administration of a transgene not a transgenic product. Because neither Trapnell et al. nor Smith et al. disclose using only a single administration of a vector to increase tolerance to transgenic cells or for producing transgenic product, neither Trapnell et al. nor Smith et al. can anticipate Claims 16 and 26, and their dependents.

When considered in their entirety, Trapnell et al. and Smith et al. teach away from applicants' invention as the object of both Trapnell et al. and Smith et al. is to allow for effective repeated administration of a vector. Accordingly, Claims 16, 26, and their dependents are not anticipated by Trapnell et al. or Smith et al., and thus, this rejection should be withdrawn.

Section 112 Written Description Rejections of Claims 9-12, 16-18, 23-25 and 29-32

Claims 9-12, 16-18, 23-25 and 29-32 were rejected under 35 U.S.C. § 112, ¶ 1 as failing to comply with the written description requirement. Claims 9-10 and 24 have been canceled. Claims 16, 25, 26, 28, 29 and 32 have been amended.

With respect to Claim 16, the Examiner has asserted that the original disclosure fails to recite the limitation of "wherein said immunosuppressant is administered before . . . administration of the transgenic product into the mammal." As the Examiner has recognized the claim should read "wherein said immunosuppressant is administered before . . . administration of the *transgene* into the mammal" and has been amended accordingly.

With respect to Claim 29, the Examiner asserts that there is no disclosure in the specification to support a method of *ex vivo* administration. Applicants traverse respectfully this rejection. First, the specification describes an *ex vivo* administration of genetic material. For example, on page 4, lines 26-31 of the specification, applicants describe that "genetic material is inserted into cells *in vitro* or *in vivo* in the form of one or more nucleic acid chains. This is carried out, for example, with the aid of viral vectors, such as adenoviruses, retro viruses or herpesviruses, or other methods, for example by means of transfection, by direct injection, by gene gun, or with the aid of liposomes, virosomes or receptor-mediated transport systems." (emphasis added). If the genetic material is inserted *in vitro* as described in the specification, then to get the genetic material into the mammal, an *ex vivo* method must be employed. Second, as evidenced by the attached publications, several *ex vivo* methods were known in the art at the time the application was filed. See Yang et al., "Gene Therapy for Central Nervous System Injury: the Use of Cationic Liposomes," J. of Neurotrauma, 14(5):281-297 (1997) ("Ex vivo gene transfer into the CNS is relatively mature in animal studies following more than a decade of experimental studies."); Robbins et al., "Viral Vectors for Gene Therapy," Trends in Biotechnology, 16(1):35-40 (Jan. 1998) (Summarizes the use of different viral vectors for *ex vivo* and *in vivo* gene transfer.); Mahvi et al., "DNA Cancer Vaccines: A Gene Gun Approach," Immunology Cell Biology, 75(5):456-60 (1997) (Summarizes the use of a gene gun in gene transfer for use in ex vivo gene therapy protocols.); Onyia et al., "Osteoprogenitor Cells as Targets for Ex Vivo Gene Transfer," (Describes ex vivo protocol for transferring genes by transducing osteoprogenitor cells with recombinant retro virus.).

Section 112 Enablement Rejections of Claims 9-12, 16-18, and 23-32

Claims 9-12, 16-18, and 23-32 were rejected under 35 U.S.C. § 112, ¶ 1 as failing to comply with the enablement requirement. Claims 9-10 and 24 have been canceled. Claims 16, 25, 29, and 32 have been amended. Claims 16 and 26 have been amended to make clear that Claim 16 is directed to an *in vivo* method for introducing a transgene into a mammal whereas Claim 26 is directed to an *ex vivo* procedure for introducing transgenic cells into a mammal.

The Examiner has asserted a number of grounds for the enablement rejection including reasserting some rejections from prior Office Actions. Applicants have grouped each rejection by subject matter and will address each ground separately below.

Administration of transgenic product

The Examiner asserts that there is no support in the specification as filed for the administration of the transgenic product into the mammal. Claim 16 has been amended to replace "transgenic product" with "transgene." Accordingly, it is clear that the claim is directed to administration of a transgene into a mammal, and thus, this rejection is now moot.

Ex vivo method

With respect to claims 16, 26 and their dependents, the claims, as amended, are directed to an in vivo procedure. Accordingly, this rejection should not apply to these claims. With respect to Claims 29-32, as discussed with respect to the written description rejection above, applicants have described using an *ex vivo* method. In particular, on page 4, lines 26-31 of the specification, applicants describe that "genetic material is inserted into cells *in vitro* or in vivo in the form of one or more nucleic acid chains. This is carried out, for example, with the aid of viral vectors, such as adenoviruses, retro viruses or herpesviruses, or other methods, for example by means of transfection, by direct injection, by gene gun, or with the aid of liposomes, virosomes or receptor-mediated transport systems." Thus, applicants have provided a number of different methods for inserting the genetic material into a mammal's cells *in vitro*. Moreover, *ex vivo* methods were well known in the art at the time of filing (see the citations provided above and the attached publications). Given the disclosure in the specification and the *ex vivo* methods known in the art, one of ordinary skill in the art would be able to practice the claimed invention.

Treatment of disease

The Examiner asserts that the Claims 26-32 are not enabled for treating diseases. Applicants have canceled the claims directed to a method of gene therapy by prior amendment. The remaining claims are directed to increasing the tolerance to transgenic cells and to producing a transgenic product. Accordingly, this rejection is now moot.

Route of administration and dosage

The Examiner asserts that the claims are not supported for administering the immunosuppressant by methods other than intravenously or intraperitoneally To support this rejection, the Examiner has cited to Gruber et al. for the proposition that DSG is known to be ineffective as immunosuppressant when given orally. Applicants have canceled Claim 9 which was directed to particular routes of administration including oral administration. Applicants also note that Claim 26 already recites administration intravenously or intraperitoneally, and thus, this rejection should not apply to Claim 26 and its dependents.

The test of enablement requires a determination as to whether one of skill in the art can practice the claimed invention without undue experimentation. Applicants submit undue experimentation would not be required to practice the present invention. The claimed invention is a method for expressing a transgenic product in a mammal and a method for increasing the tolerance of a mammal to transgenic cells. One skilled in the art, seeking to practice such a method on a certain mammal containing a certain transgene can simply use the assay described on page 8 of the application to determine which immunosuppressant to use and in what amount, what route of administration to use, and how long such an immunosuppressant should be applied.

Moreover, as long as "a statement of utility in the specification contains within it a connotation of how to use, and/or the art recognizes that standard modes of administration are known and contemplated, 35 U.S.C. 112 is satisfied." MPEP § 2164.01(c). Here, the specification provides examples of administering the immunosuppressants, DSG, cyclosporin A, and FK 506, intraperitoneally, at given dosages, for various periods of time. Applicants are not required to submit information that can be determined by routine experimentation, even if a considerable amount of experimentation would be needed. See MPEP § 2164.06 ("The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed."). Here, by routine experimentation, one of skill in the art by using the examples in

the specification, and the knowledge in the art, would be able to determine a suitable combination of immunosuppressant, dosage, and route of administration to achieve the claimed level of transgenic product. Accordingly, practicing the claimed invention does not require undue experimentation.

Any experimentation necessary to determine an effective dosage or route of administration of an immunosuppressant would be routine for one skilled in the art in view of the more than reasonable guidance provided and the extensive guidance in the scientific literature on immunosuppressant administration. It cannot be the case that Applicants after discovering the lasting effects of immunosuppressant administration on the levels of transgenic products must provide examples of administering the immunosuppressant in different dosages and by different routes of administration. Such a requirement would be unduly burdensome on applicants and exceed the requirements set forth in 35 U.S.C. § 112. Accordingly, Applicants respectfully request that this rejection be withdrawn.

Transgenes

The Examiner seems to be asserting that the pending claims are not enabling for transgenes other than the ones disclosed in the Examples. The Examiner asserts that the transgene has an effect on expression levels because Example 1 and 2 have different results. First, the results of Examples 1 and 2 are very similar. As the Examiner notes, Example 1 shows 10% of maximal expression on the 42nd day after vector administration. Whereas, Example 2 shows 36% of maximal expression on the 30th day after vector administration – not 50% as the Examiner asserts – (10001250/27775000 x 100) and 8% expression on the 60th day after vector administration (213500/27775000 x 100). Accordingly, as Example 1's expression falls within this range, the Examples are consistent. Second, applicants fail to understand the basis of the Examiner's assertion. In both Examples 1 and 2, a proper control was used (no immunosuppressant), and the level of product expressed by the mice administered both a vector and an immunosuppressant is much higher than the control. Even if it were the case that the greater expression of AAT in Example 2 as compared to the expression of beta-galactosidase in Example 1 may be attributed to properties of AAT, it still can not be ignored that the level of expressed AAT in the mouse treated with DSG is

significantly greater than the level of expressed AAT in the mouse not treated with DSG.

Vector

Similar to the argument above, the Examiner seems to be asserting that it is possible that the increased tolerance caused by DSG treatment in applicants' Example 1 may be due to a different vector being used for the DSG-treated mice because the vectors used in Example 1 are based on the vectors in Bett et al. and because Bett et al. disclosed more than one adenoviral vector. Again, this assertion is without merit because the proper controls were used. As both the DSG-treated group and the control group are administered the same adenoviral vector, it is clear that the increased expression of beta-galactosidase was due to the treatment with DSG.

Accordingly, in view of applicants' amendments and the remarks above, applicants request respectfully that the enablement rejections be withdrawn.

Section 112 Indefiniteness Rejections of Claims 9-12, 16-18, 23-25, and 29-32

Claims 9-12, 16-18, 23-25, and 29-32 were rejected under 35 U.S.C. § 112, ¶ 2 as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicants regard as the invention. Claims 9-10 and 24 have been canceled. Claims 11-12, 17-18, 23, and 25 depend from Claim 16. Claims 30-32 depend from Claim 29.

Claim 16

Claim 16 and its dependent claims were rejected for omitting the step of administering the transgenic product to the mammal. Applicants have amended Claim 16 to make clear that the method involves the administration of a transgene to a mammal and not a transgenic product. Accordingly, the rejection based on the administration of a transgenic product should be withdrawn.

Claim 16 was also rejected for lacking antecedent basis for the recitation "introducing into a cell of *said* mammal a transgene capable of expressing *said* transgenic product."

Applicants traverse respectfully this rejection. Both the terms "mammal" and "transgenic product" are introduced in the preamble. Applicants assert that changing the "said" to "the" does not change the structure of the claim. Moreover, replacing "said" with "a" would make the claim unclear as the method is directed to administering a transgene to the same mammal to produce the same transgenic product as indicated in the preamble. Accordingly, applicants request that this rejection be withdrawn or that the Examiner provide a citation to support this rejection.

Claim 29

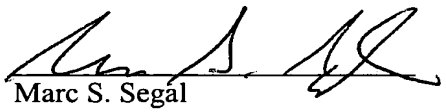
Claim 29 and its dependent claims were rejected for omitting the step of administering the transgenic cells to the mammal. Applicants have amended Claim 29 to insert the recitation "introducing said transgenic cells into said mammal" to address the Examiner's rejection. Accordingly, this rejection should be withdrawn.

Claim 29 was also rejected for lacking antecedent basis for the recitation "introducing into a cell of said mammal a transgene capable of expressing said transgenic product." Applicants have amended Claim 29 to replace "said transgenic product" with "a transgenic product." With respect to the recitation "said mammal," this refers back to the preamble as discussed above. In light of applicants' amendment, applicants request that this rejection be withdrawn.

Conclusion

In view of the proposed claim amendments and the arguments presented above, the present application is believed to be in condition for allowance and an early notice thereof is earnestly solicited. The Office is invited to contact the undersigned counsel in order to further the prosecution of this application in any way.

Respectfully submitted,



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Gene Therapy for Central Nervous System Injury: The Use of Cationic Liposomes: An Invited Review

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ABSTRACT

This paper briefly reviews general principles of gene therapy with emphasis on the therapeutic potential of cationic liposome-mediated neurotrophin gene transfer to treat central nervous system (CNS) injury. Current developments in studies of gene therapy for CNS injury are both impressive and promising. *Ex vivo* gene transfer into the CNS is relatively mature in animal studies following more than a decade of experimental studies. *In vivo* gene transfer into the CNS has gained more attention recently. Although progress has been made using viral vectors, rapid advances in transfection technologies employing cationic liposomes, together with the relatively low toxicity of these nonviral vector systems, suggest that liposomes may have significant potential for clinical applications. Although many investigators have recognized that gene therapy may be useful for treatment of certain genetic defect diseases or cancer, gene therapy for CNS injury is relatively novel. In contrast to genetic defect disorders, temporary induction of transgenes may have therapeutic applications for CNS injuries such as stroke and trauma. Employing gene transfer techniques to achieve therapeutically useful levels of expression of neurotrophins in the CNS could provide a new strategy for treatment of the traumatically injured CNS.

Key words: Gene transfer; neurotrophin; CNS injury; liposome

INTRODUCTION

During the past few years, there has been rapid growth in the concepts and techniques surrounding gene therapy, including its applications to disorders of the central nervous system (CNS) (Friedmann and Jinnah, 1993; Karpati et al., 1996; Iwamoto et al., 1996). Although it has been recognized that gene therapy has significant potential for the treatment of many inherited genetic defect diseases or cancer, gene therapy for CNS injury is relatively novel. The therapeutic potential of various neurotrophins for treating injuries of the CNS is widely appreciated. Investigators studying CNS injury have long known that administration of specific proteins such as neurotrophins could have important therapeutic potential for intervention in the pathological responses to injury and recovery of function in the injured brain (Hefti, 1986; Williams et al., 1986; Kromer, 1987; Hagg et al., 1990; Fischer et al., 1991; Fischer & Bjorklund, 1991; Rylett et al., 1993; Dekker et al., 1992, 1994; McDermot et al., in press; Dixon et al., in press). Therapeutically relevant strategies for manipulating production of these proteins could ultimately have important implications for the treatment of neuronal injury. Employing gene transfer

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techniques to achieve therapeutically useful levels of expression of neurotrophins in the CNS could provide a new strategy for intervention in the traumatically injured CNS. This paper reviews cationic liposome-mediated gene transfer for CNS injury. Rationales for this focused review include the observations that (1) blood–brain barrier opening after injury provides a specific therapeutic window for the gene transfection; (2) traumatic brain injury (TBI) is not a genetic defect disorder and permanent gene transfer may not be necessary. TBI is associated with disturbances of the blood–brain barrier, which may enhance delivery of transgenes by vectors that would otherwise have restricted access to the CNS. In contrast to genetic defect disorders, temporary induction of transgenes may have therapeutic applications for CNS injuries such as stroke and trauma. Recent studies suggest that cationic liposome-mediated gene transfection is associated with low toxicity and may be better suited for application in transient transgene expression.

This paper will briefly review the general principle of gene therapy with emphasis on the therapeutic potential of cationic liposome-mediated neurotrophin gene transfer for treating CNS injury. We will focus on studies examining the potential usefulness of cationic liposome-mediated neurotrophin gene transfer for enhancing recovery from neurotrauma. For readers who have broader interests in gene therapy, other reviews are available. For current status of general gene therapy for CNS disorders, readers can refer to the reviews by Suhr and Gage (1993); Fick and Israel (1994); Karpati et al. (1996); and Heistad and Faraci (1996). For viral vector-mediated gene therapy, readers can refer to the review by Breakefield (1993); Smith (1995); Haddada et al. (1995); Berns and Giraud (1995); and Ginsberg (1996). For nonviral vector-mediated gene therapy, readers can refer to reviews by Felgner (1993, 1996); Gao and Huang (1995); and Ledley (1995).

THE BASIC CONCEPT OF GENE TRANSFER AND GENE THERAPY

Gene therapy is a strategy that uses a gene transfer technique to transfer biologically relevant genetic material into somatic cells to treat disease. Gene transfer can use one of a number of different modalities to transfer a piece of cDNA that codes for a transgene and can be expressed after transfer into the host cell.

Based on target cell differences, gene therapy investigations can be categorized into two different approaches: *ex vivo* and *in vivo* gene transfer. The methods of transferring genes into the cells can be divided into two major categories: viral vectors and nonviral vectors. Each approach has its own advantages and disadvantages. It is too early to say any vector system is superior and, in fact, different vector systems may be better for different applications. The following section provides a more detailed review.

Ex Vivo and In Vivo Gene Transfer

Ex vivo *gene transfer. Ex vivo* gene transfer relies on genetic transfer to cultured cells that are subsequently implanted into a host organism. *Ex vivo* gene transfer uses techniques that include molecular cloning, cell culture, and graft implantation to deliver the therapeutic genes. The transgenic graft implant acts as a manufacturing reservoir, responsible for synthesizing and secreting therapeutic agents within the CNS. Some neurological deficits are confined to very small physical regions of the brain in which low levels of protein produced by small grafts may be sufficient to elicit therapeutic effects. The gene carrier cells can be obtained from cell lines or primary cell cultures. Cell line grafts have the advantage of simple preparation, are readily maintained in culture, and potentially grown in large numbers. However, the potential of tumorigenesis and graft rejection due to the heterologous nature of the cells can be problematical. Because primary cell carriers overcome the graft rejection problem, they have become favored carriers for *ex vivo* gene transfer, although they have the disadvantages of unstable transfection efficiency and deceasing transgene expression over time. In primary cell carriers, investigators can choose between neuronal and nonneuronal cells. Since it is difficult to grow and select primary neuronal cultures, most studies have been conducted in nonneuronal primary cell carriers, which include astrocytes, adrenal chromaffin cells, myoblasts, and fibroblasts. Among them, fibroblasts have been most extensively studied and characterized. Many studies using genetically modified primary fibroblast grafts have provided promising results for the use of *ex vivo* gene transfection to treat the CNS injuries (Rosenberg et al., 1988; Suhr and Gage, 1993; Tuszynski et al., 1994, 1996a,b).

In recent years, significant progress has been made in *ex vivo* gene transfer for CNS injuries. Nerve growth factor (NGF)-producing grafts have been shown to reduce behavioral deficits in rats with lesions of the nucleus basalis magnocellularies (Dekker et al., 1994). After implanting genetically modified NGF-producing fibroblasts in the aged brain, significant amelioration of memory impairment and increases in size and number of neuronal cells in the basal forebrain were observed (Chen and Gage, 1995). Also, *ex vivo* gene transfer of NGF has been shown to promote the survival of axotomized septal neurons and the regeneration of their axons in rat brain (Kawaja et al., 1992). *Ex vivo* gene transfer of NGF can

also induce robust neuritic ingrowth in unlesioned rat spinal cord (Tuszynski et al., 1994) and induce differential outgrowth of neurites in hemisection-lesioned rat spinal cord (Tuszynski et al., 1996a). Brain-derived neurotrophic factor (BDNF)-transduced fibroblasts grafted to the rat brain showed continued mRNA production for at least 2 weeks (Lucidi-Phillipi et al., 1995). Investigators have proposed that grafting of neurotrophin-producing cells may be a clinically applicable approach to treating TBI or spinal cord injuries (Suhr and Gage, 1993; Tuszynski et al., 1994, 1996a,b). Recent studies in a rat spinal cord injury model suggest that treatment with genetically engineered fibroblasts producing NGF or BDNF can accelerate recovery from TBI (Kim et al., 1996).

An advantage of *ex vivo* gene transfer is that the technique avoids many ethical problems associated with fetal tissue transplantation. Another advantage of *ex vivo* gene transfer compared to *in vivo* approaches is that the transgene can be screened and selected in host cells *in vitro* before transplanting *in vivo*. This allows a potential to graft a group of uniform and potent transgene-expressing cells to a highly localized area in the CNS. The disadvantages of *ex vivo* gene transfer include tumorigenesis and rejection when using cell line grafts, as well as grafted cell death and diminishing transgene expression associated with the use of primary cell grafts. The procedure is also relatively more complicated than *in vivo* gene transfer (Suhr and Gage, 1993).

In vivo *gene transfer. In vivo* gene transfer is a procedure in which the desired gene is directly transferred to recipient somatic cells. This purpose can be achieved by infection with viral vectors that carry the transgene or by using nonviral vectors to deliver the transgene to CNS cells. Major types of viral vector systems that have been tried for CNS *in vivo* gene transfer include retroviral vectors, herpes simplex type-1 viral vectors (HSV-1), adenoviral vectors, and adeno-associated viral vectors. Nonviral vector approaches include physical methods, receptor-mediated methods, and cationic liposome-mediated mechanisms. Investigations of nonviral vectors have recently focused on the use of cationic liposomes as DNA carriers. Advantages for *in vivo* gene transfer include simple procedures and low costs. Problems associated with *in vivo* gene transfection include the potential toxicity of viral vectors and the relatively low transfection efficiency of nonviral vectors. The targeting and transfer of the genes exclusively to desired cells is another major challenge for *in vivo* gene transfer.

Choosing an *in vivo* or *ex vivo* approach will rely on specific applications. Both approaches are still in relatively early developmental phases, and no reliable conclusions can be drawn about the relative merits of either approach at this time.

Methods of DNA-Mediated Gene Transfer

Viral vectors. Retroviral vectors are considered the most efficient vectors for stable gene transfer into mitotic mammalian cells. The retroviral genome integrates into the host chromosome of the infected cells. Retroviruses have a wide host range and can be extensively manipulated *in vitro*. Studies of developmental processes in CNS have used retroviral vectors to genetically modify cells prior to grafting (Wolf et al., 1988), as well as to immortalize primary brain cells through oncogene transfer (Evrard et al., 1990; Ishii et al., 1992). Retroviruses have also been widely used in CNS cancer gene therapy (Culver et al., 1992; Yamada et al., 1992; Ram et al., 1993; Tamiya et al., 1995). However, with the exception of some lentiviruses, retrovirus can integrate only into chromosomes of dividing cells. CNS cells do not normally divide and are not good retroviral targets.

Herpes simplex virus type-1 (HSV-1) has the ability to infect neuronal cells and a prolonged latency period in the CNS, thus suggesting the possible selective regulation of a therapeutic gene without inducing viral gene expression (Breakefield and Deluca, 1991). The HSV-1 is able to infect postmitotic cells and, in neurons, can be taken up anywhere along the cell surface (Lycke et al., 1988). The potential toxicity of HSV-1 remains a critical concern in the development of viral vectors for neurons. A defective HSV-1 vector has been developed, and the less pathogenic HSV-1 strain has been used to deliver the LacZ gene into the rat brain (Freese et al., 1990; Chiocca et al., 1990; Becker and Darai, 1995). However, disruption of normal neuronal architecture in animals treated with HSV-1 mutants has still been observed (Huang et al., 1992). HSVs can be toxic to cells even when the virus does not replicate (Johnson et al., 1992).

Adenoviral vectors have been widely used to infect rat CNS cells (Akli et al., 1993; Bajocchi et al., 1993; Davidson et al., 1993; Le Gal La Salle et al., 1993; Draghia et al., 1995). Adenovirus infection is usually associated with mild disease in humans, and the adenovirus genome is much larger and more complex than that of retroviruses (Smith, 1995). Like the HSV-1 viruses, adenoviruses are DNA viruses that can infect postmitotic cells. Infected cells were identified as microglial cells, astrocytes, and neurons [Le Gall La Salle et al., 1993]. The main advantages of adenoviral vectors are that they are capable of very efficient episomal gene transfer in a wide range of cells and tissues and they are easy to grow in large amounts. However, the potential toxicity of adenoviral vectors in *in vivo* applications is still a concern for clinical applications (Breakefield, 1993). Host pathogenic immunoresponses to the adenoviral vector may limit the repeated use of this vector (Smith, 1995).

Adeno-Associated Virus (AAV) is a defective parvovirus and is a nonautonomous virus containing linear single-stranded DNA (Burns et al., 1993; Kotin, 1994; Du et al., 1996; Doll et al., 1996). AAV is widespread in the human population, but it is not associated with any known disease. AAV can carry small sizes of DNA and integrate its DNA into the host chromosome of cells. The potential advantage of AAV vectors is that they can infect a broad range of cells and appear capable of long-term expression in nondividing cells. The vectors are structurally simple, and they may therefore provoke less of a host-cell response than adenoviral vectors. However, the main limitation is that vectors are difficult to grow in large amounts (Smith, 1995; Flotte and Carter, 1995).

In general, viral vectors hold many advantages for developing gene therapy to treat human diseases, including their relatively high transfection efficiency and potential for long-term expression. New developments using viral vectors have shown that gene transfer with therapeutically relevant genes has potential for treatment of neurological disorders such as stroke (Betz et al., 1995; Heistad and Faraci, 1996; Lawrence, 1996) and Parkinson's disease (Choi-Lundberg et al., 1997). Betz et al. (1995) reported that intraventricular injection of adenoviral vector carrying the interleukin-1 receptor antagonist protein (IL-1ra) can attenuate rat brain injury caused by permanent focal ischemia. In the gene-transfected rat, the cerebral infarct volume was reduced 64% compared to the nontreated group (Betz et al., 1995). Recent studies also reported that a herpes simplex viral vector carrying a glucose transporter gene has a protective effect on focal ischemia injury. Ischemic hemisphere exhibited a 67% neuronal survival rate after being transfected with a glucose transporter gene compared to a 32% neuronal survival rate in untransfected controls (Lawrence et al., 1996). Bohn's laboratory has reported that injection of an adenoviral vector encoding glial cell line-derived neurotrophic factor (GDNF) could support the growth and survival of dopaminergic (DA) neurons. GDNF gene transfection reduced loss of DA neurons about three-fold 6 weeks after 6-hydroxydopamine lesions as compared to nontreatment (Choi-Lundberg et al., 1997). However, the possible host immune response against viral vectors, which can reduce transgene expression, still remains an obstacle for therapeutic application. To overcome declining transgene expression caused by viral vector mediation, investigators suggest using a new generation of adenoviral vectors or nonviral vector gene transfer methods for the CNS to achieve stable transgene expression (Choi-Lundberg et al., 1997).

Nonviral vectors. Technology for the introduction of DNA into cells using nonviral vectors cells includes (1) chemical methods, (2) physical methods, (3) receptor-mediated mechanisms, and (4) cationic liposome-mediated mechanisms.

1. Chemical methods are the previously most common technique for *in vitro* DNA-mediated gene transfection. The process of transfection involves the mixture of DNA with compounds, such as calcium phosphate (Graham and VanDereb, 1973) and DEAE-dextran, followed by exposure of the recipient cells to the mixture. This method produces a chemical environment that results in DNA attaching to the cell surface. DNA is then endocytosed by as yet uncharacterized pathways and can become integrated into the host genome.
2. Physical methods of delivering DNA include microinjection (Capecchi, 1980), electroporation (Fromm et al., 1985; Wong and Neumann, 1982), and the gene gun (Yang et al., 1990). The technique of microinjection entails the direct introduction of small amounts of DNA into the nucleus of the target cell. Electroporation exposes cells to bursts of high-voltage electricity in the presence of a solution of DNA molecules in order to open up pores in the cell. The gene gun method involves the pneumatic delivery of DNA-coated gold particles into cell cultures under a vacuum (Yang et al., 1990).
3. Receptor-mediated gene transfer requires that plasmid DNA be complexed to specific polypeptide ligands that are recognized by cell surface receptors (Wu et al., 1991). These polypeptide ligand–DNA complexes can interact specifically with the receptors on the target cells and lead to receptor-mediated internalization. However, there is a potential for the DNA to be destroyed, probably by lysosomal degradation within the cell. Investigators (Wagner et al., 1992; Curiel et al., 1991) have circumvented the problem of lysosomal degradation of DNA by the concomitant use of adenoviruses or fusogenic peptides of the influenza HA protein to disrupt DNA-containing endosomes. Another approach is using the folate-mediated uptake of DNA by the cells via the folate receptors (Gottschalk et al., 1993; Lee and Huang, 1996), or using transferrin as a receptor ligand to enhance the liposome-mediated gene delivery (Cheng, 1996).
4. Cationic liposome-mediated transfection is an attractive method for introducing genetic information into eukaryotic cells and tissues because of its simplicity, decreased toxicity and increased safety, reproducibility and almost unlimited cell type or DNA size requirements (Hug and Sleight, 1991; Felgner et al., 1987, 1996; Hawley-Nelson et al., 1993; Farhood et al., 1995; Caplen et al., 1995; Lasic and Papahadjopoulos, 1995; Gao and Huang, 1995; Ledley, 1995). Although liposome-mediated gene transfection is a relatively recent addition to the repertoire of DNA transfection methodologies, liposomes have for a long time

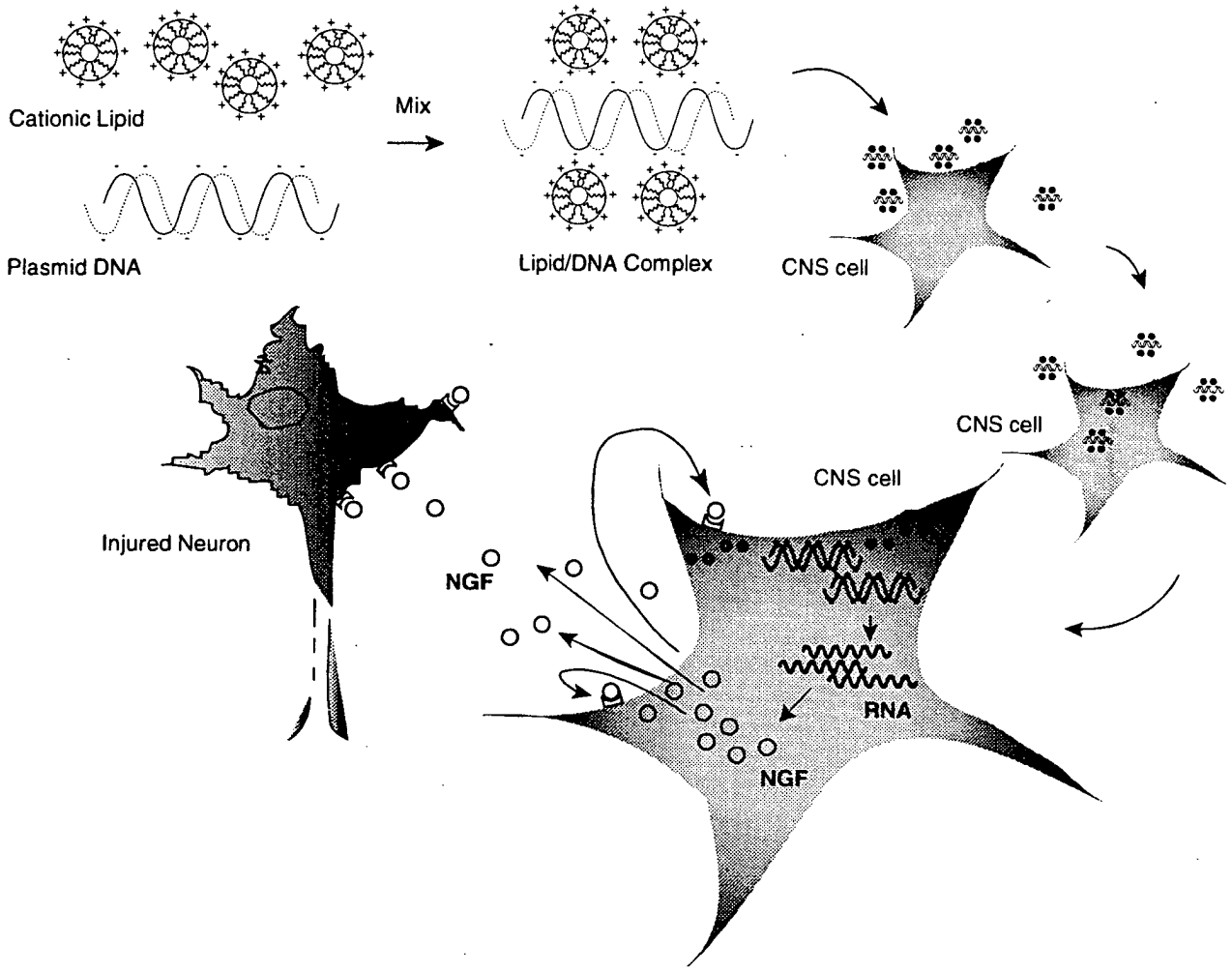


FIG. 1. Schematic representation of liposome-mediated DNA transfection. Positively charged liposomes could partially neutralize negatively charged DNA and form a net positively charged DNA–lipid complex. This positively charged DNA–lipid complex would bind to the negatively charged cell membrane and efficiently deliver functionally active genes into the cell cytoplasm. Consequently, products of the transgene could secrete proteins such as nerve growth factor (NGF) from transfected cells, bind to the injured cell membranes, and produce neurotrophic effects.

been used effectively to introduce drugs (Heath et al., 1986; Storm et al., 1988; Balazsovits et al., 1989), radiotherapeutic agents (Pikul et al., 1987), enzymes (Imaizumi et al., 1990), viruses (Faller and Baltimore, 1984; Wilson et al., 1977, 1979) and transcription factors (Debs et al., 1987) into a variety of cultured cell lines and animals. Recent progress has been made resulting in dramatic increases in transfection efficiency by cationic liposome mediation. Cationic liposomes, depending on cell line, yield from 5- to 100-fold higher transfection efficiencies than either calcium phosphate or DEAE-dextran methods of DNA delivery (Felgner et al., 1987). Cationic liposomes have also been used successfully with a number of cell types that are normally resistant to transfection by other procedures, including T-cell suspensions, primary hepatocyte cultures, and PC12 cells Muller et al., 1990). The procedure exploits the ability of cationic liposomes to transfer DNA into the cell through mechanisms not yet fully understood. One possible underlying mechanism is that positively charged liposomes partially neutralize negatively charged DNA and form a net positively charged DNA liposome complex. This positively charged DNA-liposome complex can bind to the negatively charged cell membrane and efficiently deliver functionally active genes into the cell cytoplasm (Felgner et al., 1987; Gao and Huang, 1995; Ledley, 1995; Schofield and Caskey, 1995). The mechanism of cationic liposome-mediated gene transfection is illustrated by Figure 1.

LIPOSOME-MEDIATED GENE TRANSFER FOR CNS INJURY

Recent Developments in Liposome-Mediated Gene Transfer

Recent studies have employed liposome–DNA complexes to induce gene expression in peripheral tissues and in the CNS. A single intravenous injection of cytomegalovirus (CMV)-chloramphenicol acetyltransferase (CAT) expression plasmid and DOTMA:DOPE liposomes into adult mice efficiently transfected virtually all peripheral tissues (Zhu et al., 1993). Expression of the CAT gene was still detectable 9 weeks after a single intravenous injection. Detailed analyses did not detect treatment-related toxicity in any animal. In other studies, gene expression was detected for up to 10 days after direct delivery of liposome–DNA complexes into rat kidney (Tomita et al., 1992) or after injection under the perisplanchnic membrane of adult rat liver (Kato et al., 1991). Other investigators have also concluded that the systemic injections of cationic liposome–DNA complexes into animals appear nontoxic (Stewart et al., 1992). DC-Chol liposomes have recently been tested on animal models. Toxicity studies suggested that systemic injection of DC-Chol liposomes does not cause notable abnormalities, such as inflammatory responses or negative effects on liver enzyme function (Farhood et al., 1995). Recent animal studies of intravenous injection of liposome-DNA complexes suggested that repeated intravenous injection can produce high-level systemic transfection without host-immune responses (Liu et al., 1995). Luciferase and cystic fibrosis transmembrane conductance regulator (CFTR) gene expression could be detected in mouse lung homogenate for at least 4 weeks after liposome-mediated transfection into the trachea. A recent report from a clinical trial of DC-Chol liposome-mediated CFTR gene transfer to cystic fibrosis patients indicated a partial restoration of the deficit in the patient. No adverse clinical effects were observed (Caplen et al., 1995).

The *E. coli* β-galactosidase reporter gene has been used to study liposome-mediated transfection in low-density cultures of rat hippocampal neurons. Transfection efficiency was less than 1% of the whole cell population, and the small fraction of transfected cells was mostly neuronal (Drazba and Ralston, 1993). Cationic lipids have also been used to transfect primary rodent neuronal cell cultures with a gene coding for β-galactosidase (Jiao et al., 1992). These cells were subsequently transplanted into host brains of rats. β-galactosidase was detected in host brains for 1 to 2 months after transplantation. Liposomes have been used to transfect neurons from the embryonic brain of *Xenopus in vivo* with a vector expressing luciferase. At 28 days posttransfection, brains continued to exhibit significant luciferase activity, suggesting that some of the DNA may have become integrated into the host genome of some cells (Holt et al., 1990). β-galactosidase DNA complexed with liposomes and directly injected into brains of 1-week-old mice has been reported to be incorporated and expressed by brain cells for up to 9 days postinjection (Ono et al., 1990). A number of cells that expressed the introduced gene were detectable near the ventricles. However, this study did not clarify cell types incorporating and expressing the injected cDNA. A recent report further demonstrated that injection of liposome–DNA complexes into the mouse brain can induce the reporter gene expression for at least 21 days posttransfection (Roessler and Davidson, 1994). A continuous intracerebral infusion of liposome–DNA complexes has been reported to be a more efficient means of transferring the therapeutic genes into the rodent brain (Zhu et al., 1996). Significant progress has been made in *in vivo* applications of cationic liposome-mediated gene transfection. Recent studies have suggested that liposome-mediated gene transfer with therapeutically relevant genes has potential for treatment of neurological disorders such as Parkinson's disease (Cao et al., 1995) and epileptic seizures (Zhang et al., 1997). Lipofectin-mediated tyrosine hydroxylase (TH) gene has been transfected in animal models of Parkinson's disease by direct injection in rat striatum. Behavioral tests demonstrated a significant decrease in rotational behavior after TH gene transfer in nigrostriatal pathway lesioned rats (Cao et al., 1995). The cholecystokinin (CCK) gene was transfected by liposome-mediation in a rodent model of audiogenic seizures. Following intracerebroventricular injection of lipofection and CCK cDNA complexes, increased CCK mRNA was detected and suppression of audiogenic seizures was observed (Zhang et al., 1992, 1997).

Recently, substantial progress has been made to improve cationic liposome-mediated gene transfection efficiency. New techniques have achieved expression levels much higher than previously reported, approaching transgene expression levels achieved with adenoviral vector-mediated gene transfection (Felgner, 1996). Cationic liposomes have been considered promising nonviral vectors for gene therapy (Radler et al., 1997). Polylysine has been employed to condense the plasmid DNA before the DNA is complexed with liposomes. This polylysine precondensation was particularly effective when transfecting the cells in the presence of serum, which potentially benefits the *in vivo* gene transfection (Vitiello et al., 1996). Another recent report suggested that liposome-mediated gene transfection in HeLa cells can achieve 98–100% efficiency in the presence of transferrin. The mechanism of this enhancement of gene transfection is not clear, but one possible explanation is that it facili-

TABLE 1. CURRENT COMMERCIALLY AVAILABLE CATIONIC LIPOSOMES

Trade name	*Composition*	*Manufacturer*
Lipofectin	DOTMA/DOPE	GIBCO BRL
LipofectAMINE	DOSPA/DOPE	GIBCO BRL
CELLFECTIN	M-TPS/DOPE	GIBCO BRL
DMRIE-C	DMRIE/Cholesterol	GIBCO BRL
Transfectam	DOGS	Promega
Tfx	Fusogenic Lipid/DOPE	Promega
DOTAP	DOTAP	Boehringer Mannheim

tates the entry of DNA through the transferrin-receptor mediation (Cheng, 1996).

To facilitate the studies of nonviral vector-mediated gene transfer, the National Institutes of Health (NIH) recently picked Gary Nabel's group at the University of Michigan, Ann Arbor, as one of three national gene vector laboratories to develop nonviral vectors for use by physicians around the country (Science News, 1995). This is the first time that NIH has made such a significant investment in the study of nonviral vector-mediated gene transfection for potential medical therapies. This decision should significantly facilitate the developmet of nonviral vector-mediated gene transfer.

Rationale for Using Cationic Liposome-Mediated Gene Transfer to Treat CNS Injury

Cationic liposomes are attractive alternatives to viral vector systems. Interest in liposomes as plasmid DNA carriers was rejuvenated by the development of cationic liposomes, which can condense DNA and increase transfection efficiency both *in vitro* and *in vivo* (Felgner et al., 1987; Felgner, 1996; Nabel, 1993; Zhu et al., 1993; Gao and Huang, 1995). Cationic liposomes have several attractive features as DNA carriers. First, they are less toxic and nonimmunogenic. One of the advantages of synthetic carriers is their relative safety in comparison to viral vectors. Liposomes present little or no toxicity to the host (Felgner, 1993; Gao and Huang, 1995). Liposomes are generally nonimmunogenic and are not biohazardous, as they are made from biodegradable lipids. Liposomes have been used to deliver a variety of agents in experimental studies (Hug and Sleight, 1991; Nabel et al., 1992a,b; Mori and Fukatsu, 1992; Stewart et al., 1992; Zhu et al., 1993). Successful clinical trials examining the effectiveness of liposome-mediated drug delivery have been completed (Lopez-Berestein et al., 1985; Coune, 1988). These studies did not detect any significant toxicity. Liposomes also have been investigated as DNA carriers in both experimental and human gene therapy protocols. There are no reports of significant toxicity from these studies (Caplen et al., 1995). Second, the procedure is simple. Liposomes are relatively simple to prepare and can be administered into the body by several different routes. Third, transfection can be done in many different cell types, including postmitotic cells: In addition to transfecting a wide variety of rapidly growing cells, liposomes can also transfect postmitotic, nondividing cells (Gao and Huang, 1995; Felgner, 1993, 1996). Fourth, there is no

TABLE 2. COMPARISON OF GENE TRANSFER TECHNIQUES

Method	*Advantages*	*Disadvantages*
Viral vectors	High transfection efficiency; high transgene expression levels; transfection mechanism is well understood; HSV-1 and adenoviral vectors have been shown to be useful for CNS gene transfer.	Cellular toxicity; host immunopathologic responses; replication risks; DNA size limitation; complex procedures.
Cationic liposome	Transfects broad host cell range including CNS cells; low cellular toxicity; no DNA size limitation; no host inflammatory response; simple procedures.	Low transfection efficiency in vivo with current commercially available liposomes; transfer mechanisms not fully understood.

DNA size limitation. Liposomes can be used to deliver any type (linear or supercoiled) of DNA (or RNA) with practically no size limitation of the nucleic acids (Gao and Huang, 1993a,b; Felgner, 1993). Cationic liposomes can complex up to almost 100% of DNA for transfection (Zhou et al., 1992). Fifth, liposomes can be designed to include necessary elements for controlled and targeted gene delivery (Lee and Huang, 1996). Specific tissues and cell types could be targeted *in vivo* by several approaches. These include using promoter–enhancer elements that are tissue- and cell-type specific, administration of the plasmid regionally into selected tissue compartments (Holt et al., 1990) and coupling a targeting ligand to the liposomal surface (Debs et al., 1987; Lee and Huang, 1996). Although the physiochemical properties of the liposome–DNA complex and its interaction with cells are not fully understood, the results of successful transfection of plasmid DNA are compelling. Future development of liposome-mediated gene transfection will continue to improve transfection efficiency and transfection specificity (Lasic and Papahadjopoulos, 1995; Gao and Huang, 1995). Sixth, transient and low levels of transfection may not compromise gene therapy for CNS injury. Cationic liposome-mediated gene transfection produces transient expression that may limit its application for certain genetic defect disorders. However, liposomal transfection of neurotrophin genes may still be useful for treatment of CNS injury by blunting transient pathological processes and/or facilitating recovery. Liposome-mediated gene delivery also produces a relatively low level of expression, which may be a concern for certain gene therapy protocols such as cancer therapy. However, we have reported that a 3–4% transfection efficiency of liposome-mediated NGF cDNA transfection in septohippocampal cell cultures can produce almost 10-fold increases of secreted NGF protein (Yang et al., 1994a,b). After transferring supernatant containing a secreted form of NGF to PC12 cell cultures, significant neurite growth was also observed (Yang et al., 1994b). Further studies using the same transfection paradigm demonstrated the rescue of neurofilament loss caused by neuronal injury (Hayes et al., 1995). Thus, liposome-mediated neurotrophin gene transfection may be clinically applicable. Furthermore, improved designs of liposomes are continuously being developed for enhanced transfection efficiency *in vitro* and *in vivo* (Lasic and Papahadjopoulos, 1995; Gao and Huang, 1995).

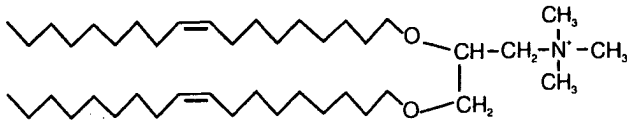
The opening of blood–brain barriers after injury may facilitate gene transfer. The presence of the blood–brain barrier significantly confounds the choice of routes of administration in gene transfection studies. Importantly, severe brain injury may provide unique opportunities for therapeutic applications of gene transfection. Ventricular cannulae are frequently used in clinical management of head injury patients and could be used to administer liposome–DNA complexes. In addition, the breakdown of the blood–brain barrier following TBI offers the opportunity for enhanced access to the CNS by cDNA-liposome complexes following injury. Although liposomes have been reported to cross the blood–brain barrier, other studies have suggested that uptake of liposomes in the normal brain is quite limited (Micklus et al., 1992).

Comparison of Lipofectin versus DC-CHOL and Assessments of Liposomal Toxicity

In recent years, many cationic liposomes with different properties have been developed. Commercially available cationic liposomes are listed in Table 1. The first cationic lipid used for transfection was lipofectin™ N-[1-(2,3-dioleyloxy) propyl]-N,N,N,-trimethylammonium chloride (DOTMA) in a liposome formulation with dioleoyl phosphatidyl-ethanolamine (DOPE) (Felgner et al., 1987). Lipofectin™ has been the most extensively used of all commercially available liposome formulations, a fact that contributed to our laboratory's initial interest in lipofectin™. DC-Chol, which was recently developed in Dr. L. Huang's laboratory, has a relatively high transfection efficiency and low toxicity (Singhal and Huang, 1994; Gao and Huang, 1995). This liposome has a tertiary amino group and a carbamoyl bond that can be hydrolyzed by cellular esterases, making it biodegradable and less toxic (Singhal and Huang, 1994). Using DC-Chol liposome as a carrier for cDNA in our *in vitro* and *in vivo* studies, we have found that DC-Chol liposomes have a relatively higher transfection efficiency and less toxicity than lipofectin. Recently, DC-Chol liposomes were approved by the FDA for a clinical trial for cancer immunotherapy (Nabel et al., 1992b, 1993). DC-Chol liposomes have also been approved in Great Britain for clinical trials of gene therapy for treating cystic fibrosis (Caplen et al., 1995). The structures of cationic lipids in lipofectin and DC-Chol liposomes are illustrated in Figure 2.

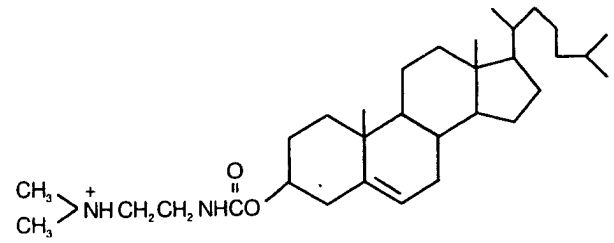
Although the use of liposomes has not been associated with significant toxicity over a wide range of dosing protocols, treatment of cells in culture with sufficiently high levels of liposomes can result in cell lysis (Felgner et al., 1987). Different cell lines may show varying degrees of susceptibility. Therefore, it is important for investigators to conduct dose toxicity studies in individual systems being employed. Experiments have shown that cultured rat septohippocampal cells, 80–90% confluent (2.8×10^5 cells per 15-mm plate), do poorly when exposed to 5 μl or more of lipofectin per well. Lysis may take up to 12 h to occur, although signs of cytotoxicity can be detected at earlier posttransfection times. However, increasing

Lipofectin™



N- [1-(2,3-dioleyloxy)propyl]-N,N,N,-trimethylammonium chloride (DOTMA)

DC-Chol



3β[N-(N',N' -dimethylaminoethane)carbamoyl] cholesterol (DC-Chol)

FIG. 2. Structures of cationic lipids in liposomes.

concentrations of lipofectin up to 3 μl/well was associated with increased transfection efficiency (Yang et al., 1994a, 1995b). *In vitro* experiments to date in our laboratory suggest that DC-Chol liposomes have significantly less toxicity at concentrations associated with transfection efficiency substantially greater than those produced by lipofectin (unpublished observation). In addition, it will also be important to consider the endpoints employed in assessments of toxicity. For example, lipofectin-mediated transfection of cDNA for BDNF can enhance the recovery of neurofilament proteins following injury to primary septohippocampal cultures (Hayes et al., 1995). However, lipofectin can also lower levels of neurofilament proteins in uninjured culture systems (unpublished observations). Therefore, optimizing the liposome concentration is critical to successful transfection.

Rationale for the Initial Choice of Transfected Genes (β-Galactosidase, NGF, and BDNF) and the Use of the Cytomegalovirus Promoter

β-*Galactosidase*

The use of β-galactosidase cDNA offers numerous advantages in initial transfection experiments determining the optimal proportions of cationic liposomes and cDNA. β-galactosidase cDNA codes for a bacterial enzyme that is not found in humans. Functional expression of the enzyme permits conversion of the colorless substrate into a bright blue precipitate, clearly labeling successfully transfected cells. This procedure is much less costly than determining transfection efficiency via *in situ* hybridization and immunohistochemical methods.

NGF and BDNF

There are numerous proteins potentially capable of enhancing CNS recovery from injury, and a number of observations suggest that the neurotrophins NGF and BDNF would be useful molecules for initial assessments of the therapeutic potential of gene transfection in TBI. NGF and BDNF have several attractive features. First, enhancement of cytoskeletal proteins by neurotrophins has been reported previously. NGF can initiate and maintain neurite outgrowth in rat pheochromocytoma (PC12) cells (Greene and Tischler, 1982). NGF is also associated with increased levels and enhanced phosphorylation of specific MAPs and with enhanced phosphorylation of β-tubulin (Aletta et al., 1988, 1990). Thus, NGF may play an indirect role in cytoskeletal stabilization. Recent studies also indicate that maintenance of hippocampal cultures in the presence of DNF results in significant increases in neurofilament protein (Ip et al., 1993), an observation our laboratory has exploited in *in vitro* studies (Hayes et al., 1995). A second feature is the protection of cholinergic neurons: Exogenous supplementation of BDNF and NGF has been reported to spare septal cholinergic neurons from death and degeneration following injury. NGF prevents degeneration of septal cholinergic neurons following fimbria lesions or transection (Hefti, 1986; Williams et al., 1986; Morse et al., 1993). NGF and BDNF increase survival of septal cholinergic neurons (Alderson et al., 1990; Winn et al., 1994) and increase choline acetyltransferase (ChAT) activity both *in vitro* (Alderson et al., 1990; Le et al., 1996) and in intact animals (Rylett et al., 1993; Scali et al., 1994). A recent report further suggested that intraparenchymal grafts of cell genetically modified to produce NGF can prevent cholinergic neuronal degeneration caused by fornix transection injury (Tuszynski et al., 1996b). Thus, gene transfection to enhance the availability of NGF and BDNF in the CNS following traumatic injury may have significant therapeutic potential. A third feature is improved behavioral deficits after TBI. The rodent hippocampus is preferentially vulnerable to a variety of CNS insults including TBI and ischemia (Hayes et al., 1991). A number of observations indicate that TBI can result in disturbances of cholinergic neurotransmission associated with impaired release of acetylcholine in the hippocampus (Dixon et al., 1993). These cholinergic deficits may underlie disturbances in spatial memory performance seen in rats after TBI. Recent studies have shown that administration of exogenous NGF can reduce spatial memory deficits in rats

following TBI (Dixon et al., in press; Sinson et al., 1995, 1996). Implantation of polymer-encapsulated NGF-secreting fibroblasts has been shown to attenuate the behavioral and neuropathological deficits following quinolinic acid-induced lesions (Emerich et al., 1994). In aged rats, intraventricular NGF infusion has significantly improved spatial memory, which is considered mediated by cholinergic neurons (Fischer, 1991; Fischer et al., 1991). Also, NGF produced by genetically modified fibroblasts has been shown to reduce behavioral deficits including those involving spatial learning (Dekker et al., 1994). Recent studies suggest that NGF infusions can promote functional recovery in TBI rodent models [McDermont et al., in press].

Cytomegalovirus promoter. When the gene remains extrachromosomal, as in liposome-mediated gene transfections, optimal levels of expression are likely to be achieved using viral promoters. Furthermore, viral promoters generally function in a broad range of cell types. The relative strengths of several commonly used viral promoters have been studied in different cell lines including primary cultures of rat mammary epithelial cells, NIH 3T3 (Thompson et al., 1993), primary rat and human hepatocytes (Fang et al., 1989; Li et al., 1992), human embryo fibroblasts (Giordano et al., 1992), and brain tumor cell lines (Dellig and Seliger, 1990). These studies indicate that the cytomegalovirus (CMV) is a prudent initial choice of a viral promoter. In primary cultures of human hepatocytes, the CMV promoter yields a higher transfection efficiency than RSV and SV40 (Li et al., 1992). RSV and CMV promoters were tested for activity in proliferating and quiescent human embryo fibroblasts. Differences in promoter activity are substantial in nonreplicating cells: the efficiency of the RSV promoter is not greater than background whereas the CMV promoter is very active. Furthermore, the CMV promoter exhibits two-fold greater activity in growing cells (Giordano et al., 1991). The CMV promoter also shows high activity in glioblastoma cell lines (Dellig and Seliger, 1990).

Optimizing Different Ratios of cDNA to Liposomes Can Facilitate Transgene Expression in CNS Cells

Initial experiments have shown that the ratio of nucleic acid to liposomes during transfection is critical for optimizing transfection efficiency in cultured cells. *In vitro* studies on septohippocampal primary cell cultures suggested that highest transfection efficiency was seen in employing a 1:3 (DNA μg/liposome μl) transfection ratio, and less efficient transfection was observed with a 1:1 transfection ratio (Yang et al., 1994a). The differential efficiencies associated with varying ratios of cDNA to liposomes are consistent with the view that the higher the net positive charge of DNA-liposome complexes, the better the interaction with a negatively charged cell membrane.

Optimized Liposome-Mediated Neurotrophin Gene Transfer Can Rescue Neurofilament Loss Caused by Injury

Because of the preferential vulnerability of the hippocampus to TBI or ischemic brain injury, our laboratory has employed primary septohippocampal cell cultures to determine the ability of liposome-mediated BDNF/NGF gene transfection to facilitate recovery of neurofilament loss caused by depolarization injury. After BDNF gene transfection in cultures, reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemical staining confirmed increases in BDNF mRNA and protein expression in transfected cells (Hayes et al., 1995). Three days after calcium-dependent depolarization injury (Whitson et al., 1995), Western blot and immunohistochemical analyses detected significant loss of neurofilament proteins in nontransfected cultures, whereas BDNF gene transfection produced marked increases in neurofilament proteins following either preinjury transfection or transfection 24 h following injury. Immunohistochemical studies also detected enhanced immunolabeling of BDNF and total neurofilament protein (phosphorylated and nonphosphorylated) in injured neurons following BDNF gene transfection or administration of exogenous BDNF protein, as compared to untransfected, injured controls (Hayes et al., 1995).

Recent studies have further investigated whether cationic liposome-mediated NGF gene transfection could modulate the expression of neurofilament proteins and phosphorylation in CNS cells (Yang et al., 1996). Two days after cationic liposome-mediated NGF gene transfection in primary cell cultures, ELISA confirmed increased NGF protein expression in NGF cDNA transfected cells. At the same time, Western blot and immunohistochemical analyses detected significant increases in phosphorylated neurofilament levels but not in the total amount of neurofilament protein in NGF cDNA transfected cell cultures. These results suggest that cationic liposome-mediated gene transfection by NGF enhances neurofilament phosphorylation rather than protein synthesis.

Optimized Liposome-Mediated Neurotrophin Gene Transfer Can Enhance Recovery from Cholinergic Neuronal Deficiency after Traumatic Injury

Septohippocampal primary cell cultures have been used to determine whether or not transfection of neurotrophin cDNA–liposome complexes into CNS cells can

enhance recovery from cholinergic neuronal deficiencies (Le et al., 1996). Employing optimal transfection conditions, pCMV/NGF cDNA was transferred into the cultures. Significant increases in ChAT, the synthetic enzyme for acetylcholine, were detected following liposome-mediated NGF gene transfection. ChAT activities increased by 18% within 2 days, 41% within 4 days and 32% within 8 days after NGF gene transfection (Le et al., 1996). To examine the therapeutic effects, the same transfection paradigm was applied to traumatized septohippocampal cell cultures. The trauma, which is induced by brief calcium-dependent depolarization, caused a significant decrease of ChAT activity. However, in liposome-mediated NGF cDNA transfection groups that were transfected 1 day after TBI, ChAT activity levels were significantly higher than the injury-only group. These protective effects can also be observed by adding purified NGF in the culture medium. However, the effect was not as potent as in the pCMV/NGF cDNA transfected group (Yang et al., in preparation).

POTENTIAL DIFFICULTIES THAT NEED TO BE RESOLVED IN FUTURE CLINICAL APPLICATIONS

Transfection Efficiency and Transgene Expression Duration

The efficiency of *in vivo* gene transfection still needs to be further improved, especially for nonviral vector-mediated gene transfer. One of the most dynamic approaches in this area is the development of new nonviral vectors. Many investigators and private companies are making great efforts to develop new nonviral vectors, and there is hope that nonviral vectors with high transfection efficiency will be developed in the near future (Lasic and Papahadjopoulos, 1995). In addition to improving clinically practical routes of delivery, maintenance of appropriate durations of expression poses challenges for the application of genetic manipulations to treat CNS injury. The host immune response against viral vectors, which can curtail transgene expression, still remains problematic. The development of a new generation of viral vectors may be a solution.

Target of Transfection

Current viral and nonviral vectors cannot target the desired cells for specific gene transfer. The ideal vector would be capable of delivering the gene only to a specific group of cells in a stable and reproducible manner. It has been suggested that ligands complexed to the vector can recognize the target cell and conduct ligand-guided gene transfer.

Potential Toxicity

The potential viral toxicity and immunoreactivity caused by viral vectors and possible cellular toxic responses caused by certain nonviral vectors are significant considerations for clinical applications. Approaches to dealing with these problems include improving the vector and/or developing new vectors that will not cause subsequent cytopathic responses, as well as improving delivery methods, thereby minimizing host inflammation. Although toxicity of nonviral vectors has not been significant to date, future studies may reveal undesirable side effects not yet detected in these systems.

SUMMARY AND CONCLUSIONS

The current developments from studies of gene therapy for CNS injury are both impressive and promising. *Ex vivo* gene transfer in CNS is relatively mature and has greater than a decade of history in experimental studies. *In vivo* gene transfer in CNS has gained more attention recently. Viral vector-mediated gene transfer has major appeal due to its high transfection efficiency, and has been extensively investigated. Cationic liposome-mediated gene transfer is an even more recent development. Rapid advances in liposome technology are exciting, and its relatively low toxicity is appealing for clinical applications. Many investigators consider cationic liposome-mediated neurotrophin gene transfer to have significant therapeutic potential for CNS injury. Initial laboratory studies suggest that liposome-mediated neurotrophin gene transfer has the potential to correct injury-induced neurofilament loss and deficits in cholinergic transmission. Although initial *in vitro* studies employed cationic liposome-mediated gene transfection systems that may have considerable therapeutic applications, it will be important for future *in vivo* studies to confirm that liposome-mediated neurotrophin gene transfer can enhance functional recovery from CNS injury.

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REFERENCES

AKLI, S., CAILLAUD, C., VIGNE, E., STRATFORD-PERRICAUDET, L.D., POENARU, L., PERRICAUDET, M., KAHN, A. and PESCHANSKI, M.R. (1993). Transfer of a foreign gene into the brain using adenovirus vectors. Nature Genetics **3,** 224–228.

ALDERSON, R.F., ALTERMAN, A.L., BARDE, Y.A., and LINDSAY, R.M. (1990). Brain derived neurotrophic factor increases survival and differentiated functions of rat sepral cholinergic neurons. Neuron **5,** 297–306.

ALETTA, J.M., LEWIS, S.A., COWAN, N.J., and GREENE, L.A. (1988). Nerve growth factor regulates both the phosphorylation and steady-state levels of microtubule-associated protein 1.2 (MAP 2). J. Cell. Biol. **106,** 1573–1581.

ALETTA, J.M., TSAO, H., and GREENE, L.A. (1990). How do neurites grow? Clues from NGF-regulated cytoskeletal phorphoproteins. In: Trophic factors and the nervous system. Horrocks, L.A. (Eds.) New York: Raven Press, pp. 203–218.

BAJOCCHI, G., FELDMAN, S.H., CRYSTAL, R.G., and MASTRANGELI, A. (1993). Direct in vivo gene transfer to ependymal cells in the central nervous system using recombinant adenovirus vectors. Nature Gen. **3,** 229–234.

BALAZSOVITS, J.A., MAYER, L.D., BALLY, M.B., CULLIS, P.R., MCDONELL, M., GINSBERG, R.S., and FALK, R.E. (1989). Analysis of the effect of liposome encapsulation on the vesicent properties, acute and cardiac toxicities, and antitumor efficacy of doxorubucin. Cancer Chemother. Pharmacol. **23,** 81–86.

BECKER, Y., and DARAI, G. (1995). Gene transfer by viral vectors for gene therapy. J. Mol. Med. **73,** 103–105.

BERNS, K.I., and GIRAUD, C. (1995). Adenovirus and adeno-associated virus as vectors for gene therapy. Ann. N.Y. Acad. Sci. **772,** 95–104.

BETZ, A.L., YANG, G.Y., and DAVIDSON, B.L. (1995). Attenuation of stroke size in rats using an adenoviral vector to induce overexpression of interleukin-1 receptor antagonist in brain. J. Cereb. Blood Flow Metab. **15**(4), 547–551.

BREAKEFIELD, X.O., and DELUCA, N.A. (1991). Herpes simplex virus for gene delivery to neurons. New Biol. **3,** 203–218.

BREAKEFIELD, X.O. (1993). Gene delivery into the brain using virus vectors. Nature Gen. **3,** 187–188.

BURNS, J.C., FRIEDMANN, T., DRIEVER, W., BURRASCANO, M., and YEE, J.K. (1993). Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: Concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. Proc. Natl. Acad. Sci. USA **90,** 8033–8037.

CAO, L., ZHENG, Z.C., ZHAO, Y.C., JIANG, Z.H., LIU, Z.G., CHEN, S.D., ZHOU, C.F., and LIU, X.Y. (1995). Gene therapy of Parkinson disease model rat by direct injection of plasmid DNA-lipofectin complex. Hum. Gene Ther. **6,** 1497–1501.

CAPECCHI, M.R. (1980). High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. Cell **22**(2), 479–488.

CAPLEN, N.J., ALTON, E.W.F.W., MIDDLETON, P.G., DORIN, J.R., STEVENSON, B.J., GAO, X., DURHAM, S.R., JEFFERY, P.K., HODSON, M.E., COUTELLE, C., HUANG, L., PORTEOUS, D.J., WILLIAMSON, R., and GEDDES, D.M. (1995). Liposome-mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis. Nature Med. **1**(1), 39–46.

CHEN, K.S., and GAGE, F.H. (1995). Somatic gene transfer of NGF to the aged brain: Behavioral and morphological amelioration. J. Neurosci. **15,** 2819–2825.

CHENG, P.W. (1996). Receptor ligand-facilitated gene transfer: Enhancement of liposome-mediated gene transfer and expression by transferrin. Human Gene Ther. **7,** 275–282.

CHIOCCA, E.A., CHOI, B.B., CAI, W., DETUCA, N.A., SCHAFFER, P.A., DIFIGLIA, M., BREAKEFIELD, X.O., and MARTOZA, R.L. (1990). Transfer and expression of the LacZ gene in rat brain neurons mediated by herpes simplex virus mutants. New Biologist **2,** 739–746.

CHOI-LUNDBERG, D.L., J LIN, Q., CHANG, Y.-N., CHIANG, Y.L., HAY, C.M., MOHAJERI, H., DAVIDSON, B.L., and BOHN, M. (1997). Dopaminergic neurons protected from degeneration by GDNF gene therapy. Science **275**(7), 838–841.

COUNE, A. (1988). Liposomes as drug deliver; system in the treatment of infectious diseases: Potential applications and clinical experience. Infection **16,** 141–147.

CULVER, K.W., RARN, Z., WALLBRIDGE, S., ISHII, H., OLDFIELD, E.H., and BLAESE, R.M. (1992). In vivo gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. Science **256,** 1550–1552.

CURIEL, D.T., AGARWAL, S., WAGNER, E., and COTTEN, M. (1991). Adenovirus enhancement of transferin-polylusine-mediated gene delivery. Proc. Natl. Acad. Sci. USA **88,** 8850–8854.

DAVIDSON, B.L., ALLEN, E.D., KOZARSKY, K.F., WILSON, J.M., and ROESSLER, B.J. (1993). A model system for in vivo gene transfer into the central nervous system using an adenoviral vector. Nature Genetics **3,** 219–223.

DEBS, R., HEATH, T., and PAPAHADJOPOLOUS, D. (1987). Targeting of anti-Thy 1.1 monoclonal antibody conjugated liposomes in Thy 1.1 mice after intravenous administration. Biochem. Biophys. Acta **901,** 183–190.

DEKKER, A.J., RAY, W.J., THAL, L.J., and GAGE, F.H. (1994). Grafting of nerve growth factor-producing fibroblasts reduces behavioral deficits in rats with lesions of the nucleus basilis magnocellularis. Neuroscience **60,** 299–309.

DEKKER, A.J., and THAT, L. (1992). Effect of delayed treat-

ment with nerve growth factor on choline acetyltransferase activity in the cortex of rats with lesions of the nucleus basalis magnocellularis: Dose requirements. Brain Res. **584,** 5563.

DELLIG, C., and SELIGER, B. (1990). Gene transfer into brain tumor cell lines: reporter gene expression using various cellular and viral promoters. J. Neurosci. Res. **26**(3), 390–396.

DIXON, C.E., TAFT, W.T., and HAYES, R.L. (1993). Mechanisms of mild traumatic brain injury. J. Head Trauma Rehab. **8**(3), 21–32.

DIXON, C.E., FLINN, P., BAO, J., VENYA, R., and HAYES, R.L. (in press). Nerve growth factor attenuates cholinergic deficits following traumatic brain injury in rats. Exp. Neurol.

DOLL, R.F., CRANDALL, J.E., DYER, C.A., AUCOIN, J.M., and SMITH, F.I. (1996). Comparison of promoter strengths on gene delivery into mammalian brain cells using AAV vectors. Gene Therapy **3,** 437–447.

DRAZBA, I.A., and RALSTON, E. (1993). Expression of *E. coli* β-galactosidase in low density cultures of hippocampal neurons. Abstr. Neurosci. Soc. **718,** 10.

DRAGHIA, R., CAILLAUD, C., MANICOM, R., PAVIRANI, A., KAHN, A., and POENARU, L. (1995). Gene delivery into the central nervous system by nasal instillation in rats. Gene Ther. **2,** 418–423.

DU, B., WU, P., BOLDT-HOULE, D.M., and TERWILLIGER, E.F. (1996). Efficient transduction of human neurons with an adeno-associated virus vector. Gene Ther. **3,** 254–261.

EMERICH, D.F., HAMRNANG, J.P., BAETGE, E.E., and WINN, S.R. (1994). Implantation of polymer-encapsulated human nerve growth factor-secreting fibroblasts attenuates the behavioral and neuropathological consequences of quinolinic acid injections into rodent striatum. Exp. Neurol. **130,** 141–150.

EVRARD, C., BORDE, I., MARIN, P., GALIANA, E., PREMONT, J., GROS, F., and ROUGET, P. (1990). Immortalization of bipotential and plastic glio-neuronal precursor cells. Proc. Natl. Acad. Sci. USA **87**(8), 3062–3068.

FALLER, D.V., and BALTIMORE, D. (1984). Liposome encapsulation of retrovirus allows efficient superinfection of resistant cell lines. J. Virol. **49**(1), 269–272.

FANG, X.J., KEATING, A., DEVILLIERS, J., and SHERMAN, M. (1989). Tissue-specific activity of heterologous viral promoters in primary rat hepatocytes and Hep G2 cells. Hepatology **10**(5), 781–787.

FARHOOD, H., GAO, X., SON, K., YANG, Y-Y., LAZO, J.S., and HUANG, L. (1995). Cationic liposomes for direct gene transferin therapy of cancer and other diseases. Ann. NY Acad. Sci. **716,** 23–35.

FELGNER, P.L. (1993). Cationic lipid/polynucleotide condensates for in vitro and in vivo polynucleotide delivery the cytofectins. J. Lip. Res. **3**(1), 3–16.

FELGNER, P.L. (1996). Improvements in cationic liposomes for in vivo gene transfer. Human Gene Ther. **7,** 1791–1793.

FELGNER, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M., and Danielsen, M. (1987). Lipofection, A highly efficient, lipid-mediated DNA-transfection procedure. Proc. Natl. Acad. Sco. **84,** 7413–7417.

FICK, J., and ISRAEL, M.A. (1994). Gene therapy for diseases of the nervous system. West J. Med. **161,** 260–263.

FISCHER, W., BJORKLUND, A., CHEN, K., and GAGE, F.H. (1991). NGF improves spatial memory in aged rodents as a function of age. J. Neurosci. **11,** 1889–1906.

FISCHER, W., and BJORKLUND, A. (1991). Loss of AchE and NGFr-labeling precedes neuronal death of axotomized septodiagonalband neurons: Reversal by intraventricular NGF infusion. Exp. Neurol. **113,** 93–108.

FLOTTE, T.R., and CARTER, B.J. (1995). Adeno-associated virus vectors for gene therapy. Gene Ther. **2,** 357–362.

FREESE, A., GELLER, A.I., and NEVE, R. (1990). HSV-1 vector mediated neuronal gene delivery. Strategies for molecular neuroscience and neurology. Biochem. Pharmacol. **40,** 2189–2199.

FRIEDMANN, T., and JINNAH, H.A. (1993). TIB tech **11,** 156–162.

FROMM, M., TAYLOR, L.P., and WALBOT, V. (1985). Expression of genes transferred into monocot and dicot plant cells by electroporation. Proc. Natl. Acad. Sci. USA **82,** 5824–5828.

GAO, X., and HUANG, L. (1993a). Cationic liposomes and polymers for gene transfer. J. Liposome Res. **3,** 17–30.

GAO, X., and HUANG, L. (1993b). Cytoplasmic expression of a reporter gene by co-delivery of T7 RNA polymerase and T7 promoter sequence with cationic liposomes. Nucl. Acids Res. **21,** 2867–2872.

GAO, X., and HUANG, L. (1995). Cationic liposome-mediated gene transfer. Gene Ther. **2,** 710–722.

GINSBERG, H.S. (1996). The up and downs of adenovirus vectors. Bull N.Y. Acad. Med. **73**(1), 53–58.

GIORDANO, T., PAN, J.B., CASUTO, D., WATANTE, S., and ARNERIC, S.P. (1992). Thyroid hormone regulation of NGF, NT-3 and BDNF RNA in the adult rat brain. Mol. Brain Res. **16,** 239–245.

GIORDANO, T., HOWARD, T.H., COLEMAN, J., SAKAMOTO, K., and HOWARD, B.H. (1991). Isolation of a population of transiently transfected quiescent and senescent cells by magnetic affinity cell sorting. Exp. Cell Res. **992,** 993–997.

GOTTSCHALK, S., CRISTIANO, R.J., SMITH, L.C., and WOO, S.L.C. (1993). Folate receptor mediated DNA delivery into tumor cells: potosomal disruption results in enhanced gene expression. Gene Ther. **1,** 165–191.

GRAHAM, F.L., and VANDEREB, A.J. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52,** 456–467.

GREENE, L.A., and TISCHLER, A.S. (1982). PC12 pheochromocytoma cultures in neurobiological research. Adv. Cell Neurobiol. **3,** 373–414.

HADDADA, H., CORDIER, L., and PERRICAUDET, M. (1995). Gene therapy using adenovirus vectors. Curr. Top. Microbiol. Immunol. **199** (pt 3), 297–306.

HAGG, T., VAHLSING, L.H., MANTHORPE, M., and VARON, S. (1990). Nerve growth factor infusion into the denervated adult rat hippocampal formation promotes its cholinergic reinnervation. J. Neurosci. **10**(9), 3087–3092.

HAWLEY-NELSON, P., et al. (1993). LipofectAMINE™ reagent: A new, higher efficiency polycationic liposome transfection reagent. Focus **15,** 73–79.

HAYES, R.L., JENKINS, L.W., and LYETH, B.G. (1991). Neurotransmitter mediated mechanisms of traumatic brain injury: acetylcholine and excitatory amino acids. Central Nervous System Status Report: 1991. J. Neurotrauma **9,** s173–s178.

HAYES, R.L., YANG, K., WHITSON, J.S., XUE, J.J., KAMPFL, A., MU, X.S., ZHAO, X., FAUSTINELLA, F., and CLIFTON, G.L. (1995). Rescue of injury-induced loss by BDNF gene transfection in primary septo-hippocampal cell cultures. Neurosci. Lett. **191,** 121–125.

HEATH, T.D., LOPEZ, N.G., PIPER, J.R., MONTGOMERY, J.A., STERN, W.H., and PAPAHADJOPOLOUS, D. (1986). Liposome-mediate delivery of pteridine antifolates to cells in vitro: potency of methotrexate, and its a and y substituents. Biochem. Biophys. Acta **862,** 72–80.

HEFTI, F. (1986). Nerve growth factor promotes survival of septal cholinergic neurons after fimbrial transections. J. Neurosci. **8,** 2155–2162.

HEISTAD, D.D., and FARACI, M.F. (1996). Gene therapy for cerebral vascular disease. Stroke **27**(9), 1688–1693.

HOLT, C.E., GARLICK, N., and CORNEL, E. (1990). Lipofection of cDNAs in the embryonic vertebrae central nervous system. Neuron **4,** 203–214.

HUANG, Q., VONSATTEL, J.P., SCHAFFER, P.A., MARTUZA, R.L., BREAKEFIELD, X.O., and DIFIGLIA, M. (1992). Introduction of a foreign gene (*E. coli* lacZ) into rat neostriatal neurons using herpes simplex virus mutants: A light and electron microscopic study. Exp. Neurol. **115,** 303–316.

HUG, P., and SLEIGHT, R.G. (1991). Liposomes for the transformation of eukaryotic cells. Biochem. et Biophys. Acta **1097,** 1–17.

IMAIZUMI, S., WOOLWORTH, V., FISHRNAN, R.A., and CHAN, P.K. (1990). Liposome-entrapped superoxide dismutase reduces cerebral infarction in cerebral ischemia in rats. Stroke **21,** 1312–1317.

IP, N.Y., LI, Y., YANCOPOULOS, G.D., and LINDSAY, R.M. (1993). Cultured hippocampal neurons show response to BDNF, NT-3 and NT4, but not NGF. J. Neurosci. **13,** 3394–3405.

ISHII, K., INOUE, H., HAKURA, A., ARAKI, W., and MASUDA, T. (1992). Immortalization of fetal mouse brain glial cells by human papillomavirus type 16 E7 genes. Cell Struct. Func. **17**(3), 197–202.

IWAMOTO, Y., YANG, K., MU, X.S., XUE, J.J., CLIFTON, G.L., and HAYES, R.L. (1996). BDNF cDNA following liposome-mediated gene transfer in intact and traumatically injured rat brain. NeuroReport **7,** 609–612.

JIAO, S., ACSADI, G., JANI, A., FEIGNER, P., and WOLFF, J. (1992). Persistence of plasma DNA and expression in rat brain cells in vivo. Exp. Neurol. **115,** 400–413.

JOHNSON, P.A., YOSHIDA, K., GAGE, F.H., and FRIEDMANN, T. (1992). Effects of gene transfer into cultured CNS neurons with a replication-defective herpes simplex virus type 1 vector. Mol. Brain Res. **12,** 95–102.

KARPATI, G., LOCHMULLER, H., NALBANTOGLU, J., and DURHAM, H. (1996). The principles of gene therapy for the nervous system. TINS **19**(2), 49–54.

KATO, K., NAKANISHI, M., KANEDA, Y., UCHIDA, T., and OKADA, Y. (1991). Expression of hepatitis B virus surface antigen in adult rat liver: Co-introduction of DNA and nuclear protein by a simplified liposome method. J. Biol. Chem. **266**(6), 3361–3364.

KAWAJA, M.D., ROSENBERG, M.B., YOHIDA, K., and GAGE, F.H. (1992). Somatic gene transfer of nerve growth factors promotes the survival of axotomized septal neurons and the regeneration of their axons in adult rats. J. Neurosci. **12,** 2849–2864.

KIM, D.H., GUTIN, P.H., NOBLE, L.J., NATHAN, D., YU, J.S., and NOCKELS, R.P. (1996). Treatment with genetically engineered fibroblasts producing NGF or BDNF can accelerate recovery from traumatic spinal cord injury in the adult rat. NeuroReport **7,** 2221–2225.

KOTIN, R.M. (1994). Prospects for the use of adeno-associated virus as a vector for human gene therapy. Hum. Gene Ther. **5,** 793–801.

KROMER, L.F. (1987). Nerve growth factor treatment after brain injury prevents neuronal death. Science **235,** 215–216.

LASIC, D.D., and PAPAHADJOPOULOS, D. (1995). Liposomes revisited. Science **267,** 1275–1276.

LAWRENCE, M., SUN, G.H., KUNIS, D.M., SAYDARN, T.C., DASH, R., HO, D.Y., SAPOLSKY, R.M., and STEINBERG, G.K. (1996). Overexpression of the glucose transporter gene with a herpes simplex viral vector protects striatal neurons against stroke. J. Cereb. Blood Flow Metab. **16**(2), 181–185.

LE, W., YANG, K., WHITSON, J.S., CLIFTON, G.L., and HAYES, R.L. (1996). Liposome-mediated NGF gene trans-

fection increases ChAT activity in rat septo-hippocampal cell cultures. NeuroReport **7,** 710–712.

LEE, R.J., and HUANG, L. (1996). Folate-targeted, anionic liposome-entrapped polylysine-condensed DNA for tumor cell specific gene transfer. Biochemistry **35,** 1027–1036.

LEDLEY, F.D. (1995). Non-viral gene therapy: The promise of genes as pharmaceutical products. Human Gene Ther. **6,** 1129–1144.

LE GAL LA SALLE, G., ROBERT, J.J., BERRARD, S., RIDOUX, V., STRATFORD-PERRICAUDET, L.D., PERRICAUDET, M., and MALLET, J. (1993). An adenovirus vector for gene transfer into neurons and glia in the brain. Science **259,** 988–990.

LI, A.P., MYER, C.A., and KAMINSKI, D.L. (1992). Gene transfer in primary cultures of human hepatocytes. In Vitro Cell Dev. Biol. **28A,** 373–375.

LIU, Y., LIGGITT, D., ZHONG, W., TU, G., GAENSLER, K., and DEBS, R. (1995). Cationic liposome-mediated intravenous gene delivery. J. Biol. Chem. **270,** 24864–24870.

LOPEZ-BERESTEIN, G., FAINSTEIN, V., HOPFER, R., MEHTA, K., SULLIVAN, M.P., KEATING, M., ROSENBLUM, M.G., MEHTA, R., LUNA, M., HERSH, E.M., REUBEN, J., JULIANO, R.L., and BODEY, G.P. (1985). Liposomal amphotericin B for the treatment of systemic fungal infections in patients with cancer: A preliminary study. J. Inf. Dis. **151**(4), 704–710.

LUCIDI-PHILLIPI, C.A., GAGE, F.H., SHULTS, C.W., JONES, K.R., REICHARDT, L.F., and KANG, U.J. (1995). Brain-derived neurotrophic factor-transduced fibroblasts: production of BDNF and effects of grafting to the adult rat brain. J. Comp. Neurol. **354,** 361–376.

LYCKE, E., HAMARK, B., JOHANSSON, M., KROTOCHWIL, A., LYCKE, J., and SVENNERHOLM, B. (1988). Herpes simplex virus infection of the human sensory neuron: An electron microscopy study. Arch. Virol. **101,** 87–104.

McDERMONT, K.L., RAGHUPATHI, R., FERNANDEZ, S.C., SAATMAN, K.E., PROTTER, A.A., FINKLESTEIN, S.P., SMITH, D.H., and MCINTOSH, T.K. (in press). Delayed administration of basic fibroblast growth factor attenuates cognitive dysfunction following lateral fluid percussion brain injury in the rat. J. Neurochem.

MICKLUS, M., GREEIG, N., TUNG, J., and RAPOPORT, S.I. (1992). Organ distribution of liposomal formulations following intracarotid infusion in rats. Biochim. et Biophys. Acta **1124,** 7–12.

MORI, N., and FUKATSU, T. (1992). Anticonvulsant effect of DN-1417, a derivative of thyrotropin-releasing hormone, and liposome entrapped DN-1417, on amygdaloid-kindled rats. Epilepsia **33**(6), 994–1000.

MORSE, J.K., WIEGAND, S.J., ANDERSON, K., YOU, Y., CAL, N., CARNAHAN, J., MILLER, J., DISTEFANO, P.S., ALTAR, C.A., LINDSAY, R.M., and ALDERSON, R.F. (1993). Brain-derived neurotrophic factor (BDNF) prevents the degeneration of medial septal cholinergic neurons following fimbria transection. J. Neurosci. **13**(10), 4146–4156.

MULLER, S.R., SULLIVAN, P.D., CLEGG, D.O., and FEINSTEIN, S.C. (1990). Laboratory methods: Efficient transfection and expression of heterologous genes in PC12 cells. DNA Cell Biol. **9**(3), 221–229.

NABEL, E.G., GORDON, D., YANG, Z.Y., XU, L., SAN, H., PLAUTZ, G.E., WU, B.Y., GAO, ., HUANG, L., and NABEL, G.J. (1992a). Gene transfer in vivo with DNA liposome complexes: lack of autoimmunity and gonadal localization. Human Gene Ther. **3**(6), 649–656.

NABEL, G.J., CHANG, A., NABEL, E.G., PLAUTZ, G.E., FOX, B.A., HUANG, L., and SHU, S. (1992b). Immunotherapy of malignancy by in vivo gene transfer into tumors. Human Gene Ther. **3,** 17–30.

NABEL, G.L. (1993). Direct gene transfer with DNA liposome complexes in melanoma: expression, biological activity, lack of toxicity in humans. Proc. Natl. Acad. Sci. USA **90,** 11307–11311.

ONO, T., FUJINO, Y., TSUCHIYA, T., and TSUDA, M. (1990). Plasmid DNAs directly injected into mouse brain with lipofectin can be incorporated and expressed by brain cells. Neurosci. Lett. **117,** 259–263.

PIKUL, S.S.II, PARKS, N.J., and SCHNEIDER, P.D. (1987). In vitro killing of melanoma by liposome-delivered intracellular irradiation. Arch. Surg. **122,** 1417–1420.

RADLER, J.O., KOLTOVER, I., SALDITT, T., and SAFINYA, C.R. (1997). Structure of DNA-cationic liposome complexes DNA intercalation in multilamellar membranes in distinct interhelical packing regimes. Science **275,** 810–814.

RAM, Z., CULVER, K.W., WALBRIDGE, S., BLAESE, R.M., and OLDFIELD, E.H. (1993). In situ retroviral-mediated gene transfer for the treatment of brain tumors in rats. Cancer Res. **53**(1), 83–88.

ROESSLER, B., and DAVIDSON, B.L. (1994). Direct plasmid mediated transfection of adult murine brain cells in vivo using cationic liposomes. Neuroscience Lett. **167,** 5–10.

ROSENBERG, M.B., FRIEDMANN, T., ROBERTSON, R.C., TUSZYNSKI, M., WOLFF, J.A., BREAKEFIELD, X.O., and GAGE, F.H. (1988). Grafting genetically modified cells to the damaged brain: Restorative effects of NGF expression. Science **242,** 1575–1578.

RYLETT, R.J., GODDARD, S., SCHMIDT, B.M., and WILLIAMS, L.R. (1993). Acetylcholine synthesis and release following continuous intracerebral administration of NGF in adult and aged Fischer-344 rats. J. Neurosci. **13**(9), 3956–3963.

SCALI, C., CASAMENTI, F., PAZZAGLI, M., BARTOLINI, L., and PEPEU, G. (1994). Nerve growth factor increases ex-

tracellular acetylcholine levels in the parietal cortex and hippocampus of aged rats and restores object recognition. Neurosci. Lett. **170,** 117–120.

SCHOFIELD, J.P., and CASKEY, C.T. (1995). Non-viral approaches to gene therapy. Brit. Med. Bull. **51,** 56–71.

Science news (1995).: NIH picks three gene vector centers. Science **269,** 751.

SINGHAL, A., and HUANG, L. (1994). Direct gene transfer by liposomes. J. Liposome Res. **4,** 289–299.

SINSON, G., VODDI, M., and MCINTOSH, T.K. (1995). Nerve growth factor administration attenuates cognitive but not neurobehavioral motor dysfunction or hippocampal cell loss following fluid-percussion brain injury in rats. J. Neurochem. **65,** 2209–2216.

SINSON, G., VODDI, M., and MCINTOSH, T.K. (1996). Combined fetal neural transplantation and nerve growth factor infusion: Effects on neurological outcome following fluid-percussion brain injury in the rat. J. Neurosurgery **84,** 665–662.

SMITH, A.E. (1995). Viral vectors in gene therapy. Annu. Rev. Microbiol. **49,** 807–838.

STEWART, M.J., PLAUTZ, G.F., DEL-BUONO, L., YANG, Z.Y., XU, L., GAO, X., HUANG, L., NABEL, E.G., and NABEL, G.J. (1992). Gene transfer in vivo with DNA-liposome complexes: safety and acute toxicity in mice. Human Gene Ther. **3,** 267–275.

STORM, G., STEERENBERG, P.A., VANBORSSUM-WALKES, M., EMMEN, F., and CROMMELIN, D.J.A. (1988). Potential pitfalls in in vitro antitumor activity testing of free and liposome-entrapped doxorubicin. J. Pharm. Sci. **77**(10), 823–830.

SUHR, S.T., and GAGE, F.H. (1993). Gene therapy for necrologic disease. Arch. Neurol. **50,** 1252–1268.

TAMIYA, T., WEI, M.X., CHASE, M., ONO, Y., LEE, F., BREAKEFIELD, X.O., and CHIOCCA, E.A. (1995). Transgene inheritance and retroviral infection contribute to the efficiency of gene expression in solid tumors inoculated with retroviral vector producer cells. Gene Ther. **2,** 531–538.

THOMPSON, T.A., GOULD, M.N., BURKHOLDER, J.K., and YANG, N.S. (1993). Transient promoter activity in primary rat mammary epithelial cells evaluated using particle bombardment gene transfer. In Vitro Cell Dev. Biol. **29A,** 165–170.

TOMITA, N., HIGAKI, J., KANEDA, Y., and OGIHARA, T. (1992). Direct in vivo gene introduction into rat kidney. Nippon-Rinsho **50**(12), 2874–2878.

TUSZYUSKI, M.H., PETERSON, D.A., RAY, J., BAIRD, A., NAKAHARA, Y., and GAGE, F.H. (1994). Fibroblasts genetically modified to produced nerve growth factor induce robust neuritic ingrowth after grafting to the spinal cord. Exp. Neurol. **126,** 1–14.

TUSZYNSKI, M.H., GABRIEL, K., GAGE, F.H., SHUR, S., MEYER, S., and ROSETTI, A. (1996a). Nerve growth factor delivery by gene transfer induces differential outgrowth of sensory, motor, and noradrenergic neurites after adult spinal cord injury. Exp. Neurol. **137,** 157–173.

TUSZYUSKI, M.H., ROBERTS, J., SENUT, M-C., U. H-S., and GAGE, F.H. (1996b). Gene therapy in the adult primate brain: intraparenchymal grafts of cells genetically modified to produce nerve growth factor prevent cholinergic neuronal degeneration. Gene Therapy **3,** 305–314.

VITIELLO, L., CHONN, A., WASSERMAN, J.D., DUFF, C., and WORTON, R.G. (1976). Condensation of plasmid DNA with polylysine improves liposome-mediated gene transfer into established and primary muscle cells. Gene Therapy **3,** 396–404.

WAGNER, E., PLANK, C., ZATLOUKAL, K., COTTEN, M., and BIRNSTIEL, M. (1992). Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylyusiube DNA complexes: toward a synthetic cirus like gene transfer vehicle. Proc. Natl. Acad. Sci. USA **89,** 7934–7938.

WHITSON, J.S., KAMPFL, A., ZHAO, X., DIXON, C.E., and HAYES, R.L. (1995). Brief potassium depolarization decreases neurofilament proteins in CNS culture. Brain Res. **694,** 213–222.

WILLIAMS, L.R., VARON, S., PETERSON, G.M., WICTORIN, K., FISCHER, W., BJORKLUND, A., and GAGE, F.H. (1986). Continuous infusion of nerve growth factor prevents basal forebrain neuronal death after fimbria-fornix transection. Proc. Natl. Acad. Sci. USA **83,** 9231–9235.

WILSON, T., PAPAHADJOPOLOUS, D., and TABER, R. (1977). Biological properties of polivirus encapsulated in lipid vesicles: antibody resistance and infectivity in virus-resistant cells. Proc. Natl. Acad. Sci. USA **74**(8), 3471–3475.

WILSON, T., PAPAHADJOPOLOUS, D., and TABER, R. (1979). The introduction of poliovirus RNA into cells via lipid vesicles (liposomes). Cell **17,** 77–84.

WINN, S.R., HAMMANG, J.P., EMERICH, D.F., LEE, A., PALMITER, R.D., and BAETGE, E.E. (1994). Polymer-encapsulated cells genetically modified to secrete human nerve growth factor promote the survival of axotomized septal cholinergic neurons. Proc. Natl. Acad. Sci. USA **91,** 2324–2328.

WOLF, D., RICHTER-LANDSBERG, C., SHORT, M.P., CEPKO, C., and BREAKEFIELD, X.O. (1988). Retrovirus mediated gene transfer of β-nerve growth factor into mouse pituitary line AtT-20. Mol. Biol. Med. **5,** 43–49.

WONG, T.K., and NEUMANN, E. (1982). Electric field mediated gene transfer. Biochem. Biophys. Res. Commun. **107**(2), 584–587.

WU, G.Y., WINSON, J.M., SHALABY, F., GROSSMAN, M., SHAFRITZ, D.A., WU, C.H. (1991). Receptor-mediated gene delivery in vivo: partial correction of genetic analbuminemia in Nagase rats. J. Biol. Chem. **266,** 14338–14342.

YAMADA, M., SHIMIZU, K., MIYAO, Y., HAYAKAWA, T., IKENAKA, K., NAKAHIRA, K., NAKAJIMA, K., KAGWA, T., and MIKOSHIBA, K. (1992). Retrovirus-mediated gene transfer targeted to malignant glioma cells in murine brain. Jap. J. Cancer Res. **83**(12), 1244–1247.

YANG, K., FAUSTINELLA, F., XUE, J.J., WHITSON, J.S., KAMPFL, A., MU, S., ZHAO, X., TAGLIALATELA, G., PEREZ-POLO, J.R., CLIFTON, G.L., and HAYES, R.L. (1994a). Optimizing liposome-mediated gene transfer in primary rat septo-hippocampal cell cultures, Neurosci. Lett. **182,** 287–290.

YANG, K., FAUSTINELLA, F., XUE, J.J., WHITSON, J.S., KAMPFL, A., MU, X.S., ZHAO, X., TAGLIALATELA, G., PEREZ-POLO, J.R., CLIFTON, G.L., and HAYES, R.L. (1994b). Sustained expression of functional nerve growth factor in primary septohippocampal cell cultures by liposome-mediated gene transfer. Neurosci. Lett. **182,** 291–294.

YANG, K., PEREZ-POLO, J.R., FAUSTINELLA, F., TAGLIALATELA, G., and HAYES, R.L. (1995b). In vitro studies of liposome-mediated gene transfection, in Paradigms in Neural Injury. J.R. Perez-Polo (ed.). Academic Press: San Diego, pp. 290–297.

YANG, K., XUE, J.J., WHITSON, J.S., KAMPFL, A., ZHAO, X., IWAMOTO, Y., MU, X.S., FAUSTINELLA, F., CLIFTON, G.L., and HAYES, R.L. (1996). Liposome-mediated NGF gene transfection increases phosphorylated neurofilament proteins. Mol. Brain Res. **43,** 13–20.

YANG, K., BIAN-XUE, J.J., MU, X.S., QIU, Y., PEREZ-POLO, J.R., CLIFTON, G.L., and HAYES, R.L. (in preparation). Liposome-mediated NGF gene transfection recover the neurofilament and ChAT loss.

YANG, N.S., BURKHOLDER, J., ROBERTS, B., MARTINELL, B., and MCCABE, D. (1990). In vivo and in vitro gene transfer to mammalian somatic cells by particle bombardment. Proc. Natl. Acad. Sci. USA **87,** 9568–9572.

ZHANG, L.X., WU, M., and HAN, J.S. (1992). Suppression of audiogenic epileptic seizures by intracerebral injection of a CCK gene vector. NeuroReport **3,** 700–702.

ZHANG, L.X., LI, X.L., SMITH, M.N., POST, R.M., and HAN, J.S. (1997). Lipofectin-facilitated transfer of cholecystokinin gene corrects behavioral abnormalities of rats with audiogenic seizures. Neuroscience **77**(1), 15–22.

ZHOU, X., KLIBANOV, A., and HUANG, L. (1992). Improved encapsulation of DNA in pH-sensitive liposomes for transfection. J. Liposome Res. **2**(1), 125–139.

ZHU, J., ZHANG, L., HANISCH, U.K., FELGNER, P.L., and RESZKA, R. (1996). A continuous intracerebral gene delivery system for in vivo liposome-mediated gene therapy. Gene Therapy **3,** 472–476.

ZHU, N., LIGGITT, D., LIU, Y., and DEBS, R. (1993). Systemic gene expression after intravenous DNA delivery into adult mice. Science **261,** 209–211.

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Viral vectors for gene therapy

Paul D. Robbins, Hideaki Tahara and Steven C. Ghivizzani

Gene therapy is now being applied to the treatment of a wide variety of acquired and inherited diseases. One of the rate-limiting steps for successful gene therapy is the efficiency of gene transfer. A number of different viral systems are being developed for use as vectors for *ex vivo* and *in vivo* gene transfer, including retroviruses, adenoviruses, herpes-simplex viruses and adeno-associated viruses. These viral vectors have a number of specific advantages and disadvantages that make them suited to particular gene-therapeutic applications. This review will summarize the current status of the development of viral vectors for gene therapy.

Gene therapy, the treatment or prevention of disease by gene transfer, has been a rapidly developing field of research[1–4]. Over the past ten years, gene therapy has been moving quickly from the laboratory to the clinic. Although the majority of the therapeutic trials to date have been phase 1, there is reason for optimism about the future of gene therapy. Clinical responses have been noted even in these current phase-1 studies, which are designed to test safety and feasibility rather than efficacy. As the relatively new field of gene therapy evolves, it is likely that gene-therapeutic approaches will become routine and accepted methods for treating both acquired and inherited diseases.

There are two approaches that may be utilized for gene therapy – an indirect, *ex vivo* method, in which cells are modified in culture and then transplanted, and a direct, *in vivo* gene-transfer method, involving the injection of a vector. Although the identification of the appropriate therapeutic gene(s) and of the target tissue are important for successful gene therapy, the rate-limiting step is still the ability to deliver the appropriate gene efficiently to the appropriate target tissue. There are two types of vector systems used for gene-therapeutic applications – viral and nonviral. For the most part, viral vectors are more effective than nonviral vectors for achieving high-efficiency gene transfer, but they have associated problems that still hinder their application to gene therapy, such as immunogenicity, pathology, targeting and/or the duration and level of gene expression. Nonviral vectors such as liposomes and DNA conjugates are nonpathogenic, but are less effective for gene transfer *in vivo*. The current status of the viral vectors for gene-transfer applications will be discussed below.

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Retroviral vectors

There are four types of virus currently in clinical trials – retroviruses, adenoviruses, herpes-simplex viruses and adeno-associated viruses. Of these vectors, the majority of clinical trials to date have used murine leukaemia virus (MLV)-based retroviral vectors for gene transfer[5–7]. Wild-type MLV encodes three proteins, from the genes *gag*, *pol* and *env*, which are processed into a number of polypeptides important for replication, encapsidation, infection and reverse transcription. The three proteins can be provided *in trans*, allowing the generation of vectors containing only the *cis*-acting elements that are required for these processes[8]. Cell lines that express the three viral proteins stably have been generated, and are termed packaging lines[9] (Fig. 1a). These cell lines can be used to produce recombinant, replication-defective virus by either stable or transient transfection. Currently, packaging lines that give titres of greater than 10^7 infectious virus particles per ml have been developed[10]. The use of different viral-envelope proteins, such as the G protein from vesicular-stomatitis virus, has improved titres following concentration to greater than 10^9 ml^{-1} (Ref. 10).

The advantage of retroviruses is that they can stably infect dividing cells by integrating into the host DNA without expressing any immunogenic viral proteins. In theory, the integrated retroviral vector will be maintained for the life of the host cell, continuing to express the gene of interest. Their disadvantages include the facts that the MLV-based retroviral vectors require cell division for stable infection, and that their coding capacity prevents the delivery of large genes, such as that encoding dystrophin. The recent development of vectors based on lentiviruses [such as human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV) or equine infectious-anaemia virus (EIAV)], which can infect certain nondividing cells, should allow the *in vivo* use of retroviral vectors for gene-therapeutic applications. Indeed, helper-virus-free

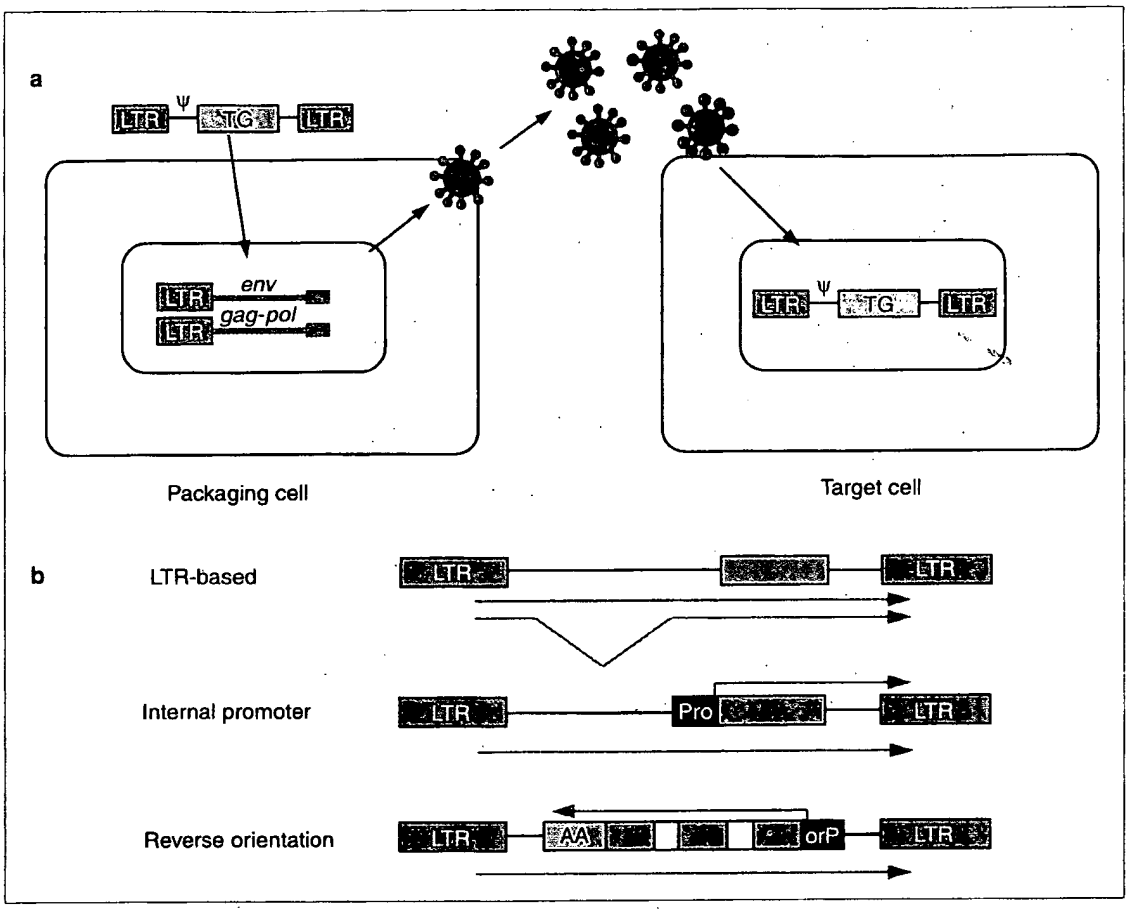

Figure 1

(a) Method for producing helper-free, replication-defective retroviral vectors. A packaging cell, genetically modified to express the retroviral *gag*, *pol* and *env* gene products stably, is either stably or transiently transfected with the retroviral vector containing LTRs encoding the therapeutic gene (TG). Because it is only the vector that contains a psi (Ψ) packaging sequence and two LTRs, only vector RNA is packaged into virions. Virus produced by the producer cells contains the retroviral-vector genome, which can stably infect and transduce the appropriate target cell. **(b)** Three different retroviral vector configurations can be used to express the gene of interest. For LTR-based vectors (top), initiation of transcription is regulated by the native retroviral promoter contained within the long terminal repeat (LTR) of the retrovirus (the site of transcription initiation and direction of RNA synthesis are indicated by the top arrow). In certain vectors, such as MFG, the gene of interest is inserted into the region of the virus normally encoding the *env* gene. For translation, the primary transcript is spliced (indicated by the bent arrow) using native retroviral splice-donor and -acceptor sequences. For vectors with an internal promoter (middle), transcription is driven primarily by an exogenous promoter sequence inserted immediately upstream of the coding region of the gene of interest. However, there is also a level of RNA synthesis initiated from the native promoter contained within the retroviral LTR. For reverse-orientation vectors, an exogenous promoter and the therapeutic gene are inserted into the vector in the opposite polarity to that of the direction of RNA synthesis from the viral LTR. Thus, transcription of the gene of interest is driven exclusively by the exogenous promoter. The positions of the murine leukaemia virus (MLV) long terminal repeats (LTR), promoter (Pro), reversed promoter (orP), polyadenylation sites (AA) and coding regions of the therapeutic gene (blue) are indicated.

stocks of recombinant HIV-based vectors have been generated that can infect a wide range of nondividing cells, including neurons, islets and muscle cells[12–14].

There are three types of retroviral vectors currently in use[5,8,15], which are similar, independent of whether the virus is of human, murine or avian origin (Fig. 1b).

(1) The long-terminal-repeat (LTR)-based vector, in which the therapeutic gene is expressed from the promoter in the 5′ LTR. An example of this type of vector is MFG, in which the therapeutic gene is inserted in place of the viral *env* gene[15,16]. The inserted gene is expressed from an LTR-driven, spliced message that has a 5′ untranslated sequence identical to the *env* message. Additional genes can be expressed from a polycistronic message in this vector using internal ribosome entry sites (IRESs). Indeed, up to three genes have been effectively expressed in MFG using two IRES elements[17,18].

(2) The internal-promoter vector, in which the therapeutic gene and/or a marker gene is expressed from an internal heterologous promoter. Additional genes can be expressed from the LTR, or the LTR can be mutated to prevent expression following infection of a target cell. The internal-promoter vectors containing

tissue-specific promoters can be used to facilitate high-level gene expression in specific cell types.

(3) The reverse-orientation vector, in which a genomic sequence containing a promoter, introns and polyadenylation signal can be inserted. For example, the β-globin gene has been inserted into a retroviral vector in the reverse orientation, so that the regulatory sequences in the 5′ promoter, introns and regions 3′ to the coding sequence can be maintained[19]. In this way, it is possible to achieve tightly regulated, tissue-specific gene expression.

Adenoviral vectors

Adenoviruses are linear double-stranded DNA viruses 30–35 kb in size that can cause upper-respiratory and eye infections in humans. The structure of human adenovirus type 5 and its different reading frames are indicated in Fig. 2. There are four different early genes expressed from the viruses after infection (E1, E2, E3 and E4), encoding polypeptides important for regulating viral and cellular gene expression, viral replication and the inhibition of cellular apoptosis. Late in infection, the major late promoter is activated, resulting in the expression of polypeptides required for encapsidation of the virus. Adenoviruses can be converted for use as vectors for gene transfer by deleting the E1 gene, which is important for the induction of the E2, E3 and E4 promoters[20]. The E1⁻, replication-defective virus can be propagated in a cell line that provides the E1 polypeptides *in trans*, such as the human embryonic kidney cell line 293. A therapeutic gene (or genes) can be inserted by recombination in place of the E1 gene; expression is driven from either the E1 promoter or a heterologous promoter (Fig. 2b).

The advantages of adenoviral vectors are that they can infect a wide variety of cell types, including non-dividing cells, and can be grown to high titres[21]. However, the viral genome remains episomal, allowing for only transient gene expression. In addition, the current first-generation E1⁻ viruses still express a low level of viral proteins after infection, resulting in a low level of viral replication. The viral gene expression can induce a CD4- and CD8-dependent immune response that reduces the duration of the gene expression *in vivo*[22,23]. In order to develop vectors that may be less immunogenic, more-defective viruses are being constructed. In particular, the deletion of some or all of the E4 open reading frames (ORFs) results in more-attenuated viruses[21,24–28]. However, certain second-generation vectors appear not to give longer-term gene expression, even though the DNA seems to be maintained. Thus, it appears that the function of one or more of the E4 ORFs may be to enhance gene expression from at least certain viral promoters carried by the virus. An alternative approach to making a more defective virus has been to 'gut' the virus completely, maintaining only the terminal repeats required for viral replication[29–31] (Fig. 2c). The 'gutted', or 'gutless', viruses can be grown to high titres with a first-generation helper virus in the 293 cell line, but it has been difficult to separate the 'gutted' vector from the helper virus.

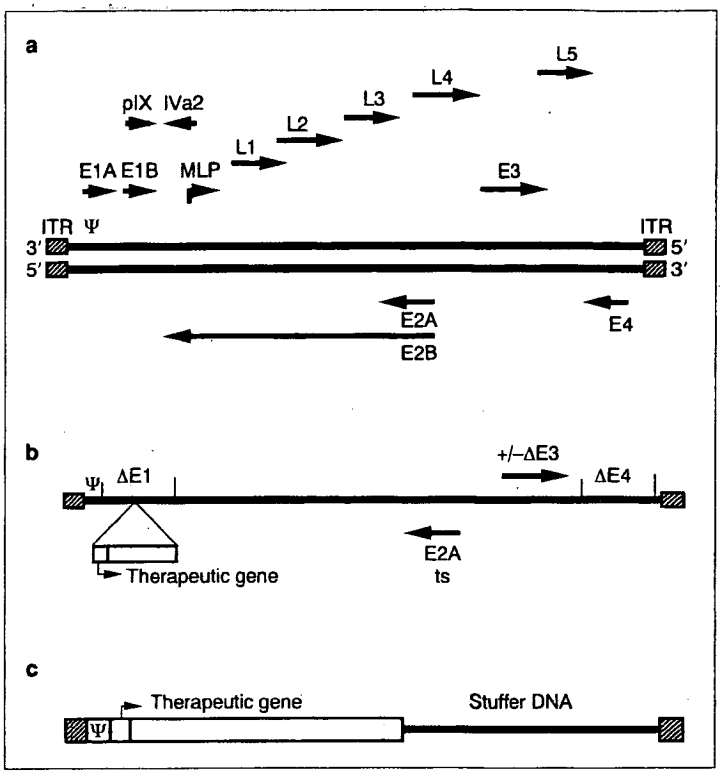

Figure 2

Structure of adenovirus type 5 (Ad5) genome. **(a)** The different transcription units for the wild-type virus are indicated by arrows. Alternative splicing leads to the RNAs for early (E), intermediate (pIX and IVa2) and late (L) genes being generated from the primary transcripts. Proteins from the early genes are required for functions such as host-cell transformation and viral-DNA replication. The late genes [transcribed from the major late promoter (MLP)] primarily encode structural proteins of the virion. Inverted-terminal-repeat (ITR) sequences function as replication origins, and the Ψ sequence is required for packaging of the viral genome. **(b)** Mutations can be introduced into the Ad5 genome to make a replication-defective virus for gene transfer. When the E1 protein is supplied *in trans* by a complementing cell line, the E1 coding region can be deleted and the therapeutic gene inserted under the regulation of a heterologous promoter. The E4 coding region has also been deleted in second-generation vectors. The E3 region, involved in blocking the immune response to the virally infected cell, can be either deleted or retained. In addition, a temperature-sensitive (ts) E2A allele has been used to make a more-defective virus. **(c)** An alternative approach to making a defective virus is to completely 'gut' it, that is, to remove all viral coding regions from the virus, leaving only the ITRs, the Ψ sequence and the therapeutic gene.

Replication-competent adenoviruses can also be used for gene therapy. For example, the E1A gene can be inserted into a first-generation virus under the regulation of a tumour-specific promoter[32]. In theory, following injection of the virus into a tumour, it could replicate specifically in the tumour but not in the surrounding normal cells. This type of vector could be used either to kill tumour cells directly by lysis or to deliver a 'suicide gene' such as the herpes-simplex-virus thymidine-kinase gene (HSV *tk*), which can kill infected and bystander cells following treatment with ganciclovir. Alternatively, an adenovirus defective only for E1B has been used specifically for antitumour

treatment in phase-1 clinical trials[33,34]. The polypeptides encoded by E1B are able to block p53-mediated apoptosis, preventing the cell from killing itself in response to viral infection. Thus in normal, nontumour cells, in the absence of E1B, the virus is unable to block apoptosis and is thus unable to produce infectious virus and spread. In tumour cells deficient in p53, the E1B-defective virus can grow and spread to adjacent p53-defective tumour cells, but not to normal cells. Again, this type of vector could also be used to deliver a therapeutic gene such as HSV *tk*.

Herpes-simplex virus

Herpes-simplex viruses I and II are large linear DNA viruses of approximately 150 kb encoding 70–80 genes (Fig. 3). The wild-type viruses are able both to infect cells lytically and to establish latency in specific cell types, such as neurons. Like adenoviruses, HSV can infect a wide variety of cell types, including muscle, tumours, lung, liver and pancreatic islets. In order to use HSV as a vector, it has to be rendered replication defective. Following infection of a cell with HSV, the expression of a small number of immediate early (IE) genes is induced by a viral transactivating protein, VP16, which is carried into the cell as part of the viral tegument. The IE genes, which include ICP0, 4, 6, 22 and 27, are themselves regulators of gene expression that are important for the induction of the early and late genes required for viral replication and encapsidation[35–38]. Mutation of ICP4 results in a virus unable to replicate except in a complementing cell line, but which still expresses the other IE-gene products; these other IE proteins are toxic to many cell types. Recently, vectors defective for ICP4, 22 and 27 have been generated that have reduced levels of toxicity and prolonged gene expression in culture and *in vivo*[35–40]. The development of vectors defective for all IE genes should, in conjunction with the appropriate complementing cell lines, allow the widespread clinical application of HSV vectors.

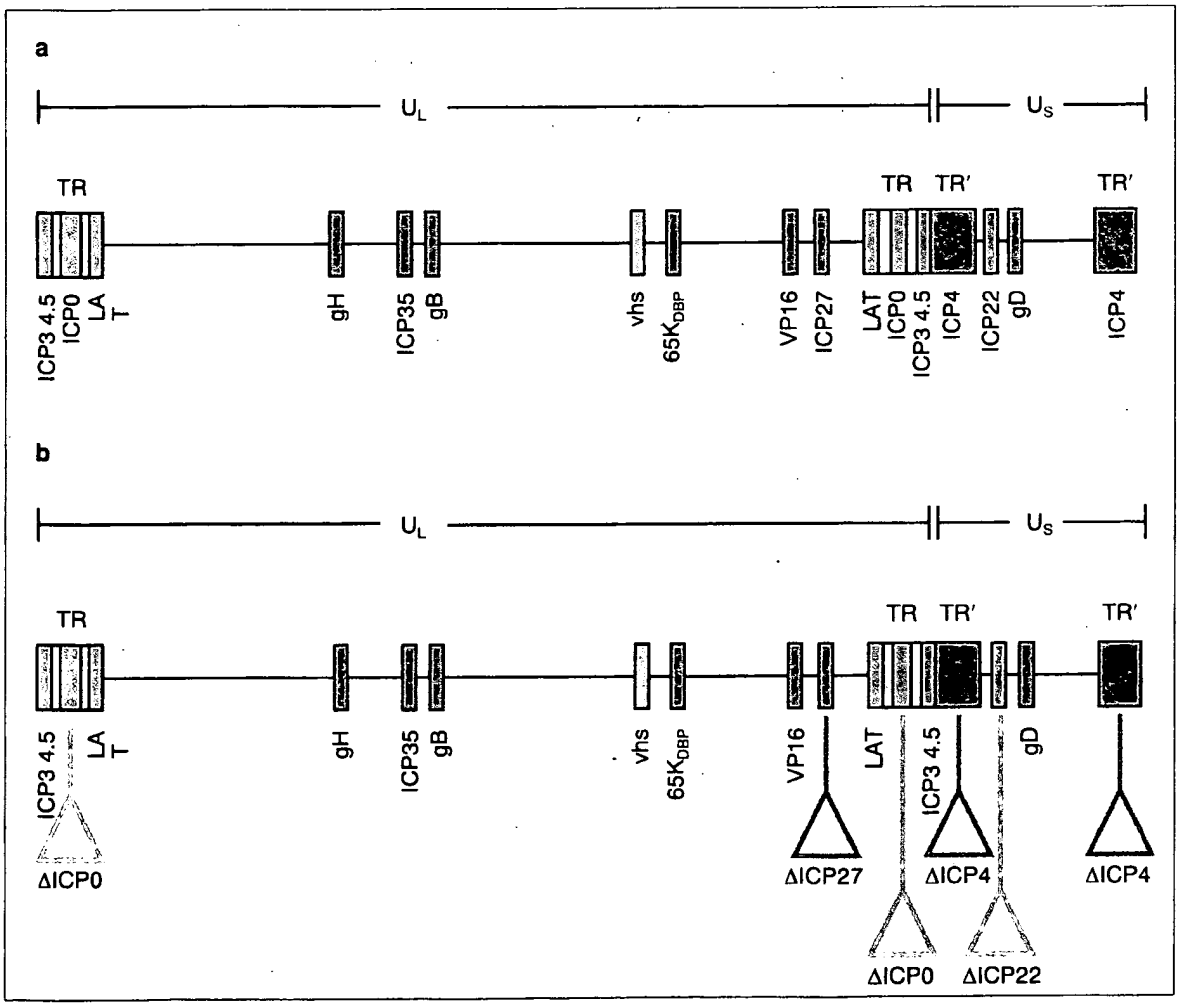

Figure 3

Structure of herpes-simplex virus. (**a**) The positions of several essential and nonessential genes are indicated, as are the unique-long (U_L) and -short (U_S) regions of the virus. (**b**) The virus can be rendered defective by deleting several of the nonessential, immediate-early genes such as ICP0, ICP4, ICP22, and ICP27. These proteins are supplied *in trans* by complementing cell lines and/or transient transfection of expression plasmids. The large size of the genome (152 kb) may allow the insertion of multiple therapeutic genes.

An alternative approach to producing infectious HSV vectors is the use of amplicons. In this approach, a plasmid containing an HSV origin of replication and packaging sequence is cotransfected with cosmids containing the HSV genome but with a defective packaging sequence[41–43]. The resulting virus contains only plasmid sequences, thereby eliminating any toxicity associated with low-level HSV-protein expression. Although this approach can generate a helper-free stock of virus, the titres are still very low.

The advantage of HSV vectors is that they have a large capacity for inserting heterologous DNA, allowing up to 50 kb to be included successfully, possibly consisting of multiple therapeutic genes. For example, four different antitumour genes have been inserted into a single HSV vector for use in cancer therapy (J. Glorioso, pers. commun.). Alternatively, HSV vectors can be used to obtain highly regulated gene expression. An RU486-hormone-regulated chimeric transcription factor has been inserted into HSV along with a promoter containing binding sites for the regulated transcription factor; specific, regulated gene expression has been observed *in vivo*.

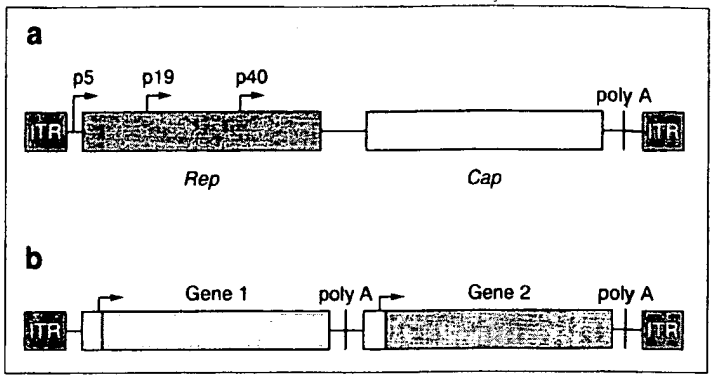

Figure 4

(a) Structure of adeno-associated virus (AAV). The position of the *rep* and *cap* genes, the inverted terminal repeats (ITR), the viral promoters (p5, p19 and p40) and the polyadenylation site (poly A) are indicated. (b) AAV can be used as a vector by inserting therapeutic genes between the ITRs.

Adeno-associated virus

Adeno-associated virus (AAV) is a member of the parvovirus family, small single-stranded DNA viruses that require a helper virus, such as adenovirus or herpes-simplex virus, for replication. AAV is a human virus, with the majority of the population being seropositive for AAV, but no pathology has been associated with it. The virus contains two genes, *rep* and *cap*, encoding polypeptides important for replication and encapsidation, respectively (Fig. 4). These two genes can be supplied *in trans* with only the inverted terminal repeats (ITRs) required *in cis* for viral replication. Therapeutic genes with the appropriate regulatory sequences can be inserted between the two ITRs, and the virus generated by cotransfection into the 293 cell line with a *rep* and *cap* expression vector and subsequent infection with a first-generation adenoviral vector[44]. The wild-type virus can be grown to high titres and is able to integrate stably into a specific region of chromosome 19 following infection[45]. However, the recombinant virus appears not to integrate site-specifically, suggesting that this integration requires the presence of the rep protein.

One of the rate-limiting steps in AAV infection appears to be the frequency of second-strand synthesis[46]. In wild-type virus infection, second-strand synthesis is stimulated by the presence of adenovirus E1 and E4 proteins; in the absence of adenovirus coinfection, unknown cellular factors appear to dictate the rate of second-strand synthesis. In certain cell types, or following treatment with DNA-damaging agents, the rate of second-strand synthesis is high[47–49]. Moreover, in several cell types, it appears that the frequency of viral integration is reduced.

Although AAV may not be suitable for gene transfer to all cell types, the degree of infection of muscle, brain and liver cells with recombinant virus is exceedingly high *in vivo*. In these cell types, stable infection and gene expression apparently occur independently of the helper virus. Injection of a β-galactosidase-containing AAV vector into muscle has resulted in β-galactosidase-positive myofibres for up to two years[50–52]. Similarly, the injection of virus into the brain also has resulted in long-term gene expression[53]. Thus, AAV may be highly suitable for the delivery of genes to specific target cells *in vivo*, without inducing an immune response to the infected cells.

Chimeric viral vectors

One of the future directions of vector development for gene therapy is the generation of chimeric vectors, which will have certain features of two or more viruses. For example, either a herpes-simplex virus or an adenovirus could be used to deliver AAV to cells in which transient expression of *rep* would allow site-specific integration of the AAV vector[54]. In this way, a large gene could be inserted into AAV. Alternatively, both the origin of replication and the *trans*-acting replication protein E1 from human papilloma virus could be inserted into a recombinant adenovirus vector; following infection, the episomal genome might be replicated and maintained by the HPV sequences. Similarly, HSV or adenovirus vectors could be used to deliver plus-strand RNA viruses, such as Sindbis, to cells[55]. Such viruses that have had their virulent capsid genes deleted could still replicate their RNA in the cytoplasm, allowing high levels of protein production[55].

Summary

Gene therapy is currently being applied to a wide range of diseases, both inherited and acquired. One, if not the, rate-limiting step to successful gene therapy is the efficiency of gene transfer. Currently, several different viral systems are being modified for use as vectors for gene therapy, including retroviruses, adenoviruses, herpes-simplex viruses, adeno-associated viruses and chimeric viruses. These viral vector systems

are currently in or approaching clinical trials, and they appear to be safe and well tolerated. Although it is likely that no single virus will be useful for all gene-therapeutic applications, with further development, viral vectors should become usable in specific gene-therapeutic applications.

References

1 Crystal, R. G. (1995) *Science* 270, 404–410
2 Evans, C. H. and Robbins, P. D. (1995) *J. Bone Jt Surg. A* 77, 1103–1114
3 Miller, A. D. (1992) *Nature* 357, 455–460
4 Mulligan, R. C. (1993) *Science* 260, 926–932
5 Miller, A. D., Miller, D. G., Garcia, J. V. and Lynch, C. M. (1993) *Methods Enzymol.* 217, 581–599
6 Miller, A. D. (1992) *Curr. Top. Microbiol. Immunol.* 158, 1–24
7 Guild, B. C., Finer, M. H., Housman, D. E. and Mulligan, R. C. (1988) *J. Virol.* 62, 3795–3801
8 Jolly, D. (1994) *Cancer Gene Ther.* 1, 51–64
9 Miller, A. D. (1990) *Hum. Gene Ther.* 1, 5–14
10 Cosset, F. L., Takeuchi, Y., Battini, J. L., Weiss, R. A. and Collins, M. K. (1995) *J. Virol.* 69, 7430–7436
11 Yee, J. K., Miyanohara, A., LaPorte, P., Bouic, K., Burns, J. C. and Friedmann, T. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 9564–9568
12 Blomer, U., Naldini, L., Verma, I. M., Trono, D. and Gage, F. H. (1996) *Hum. Mol. Genet.* 5, 1397–1404
13 Naldini, L., Blomer, U., Gage, F. H., Trono, D. and Verma, I. M. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 11 382–11 388
14 Naldini, L. *et al.* (1996) *Science* 272, 263–267
15 Robbins, P. D. *et al.* (1994) *Ann. New York Acad. Sci.* 716, 72–89
16 Dranoff, G. *et al.* (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 3539–3543
17 Zitvogel, L. *et al.* (1994) *Hum. Gene Ther.* 5, 1493–1506
18 Adam, M. A., Ramesh, N., Miller, A. D. and Osborne, W. R. (1991) *J. Virol.* 65, 4985–4990
19 Sadelain, M., Wang, C. H., Antoniou, M., Grosveld, F. and Mulligan, R. C. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 6728–6732
20 Graham, F. L. and Prevec, L. (1995) *Mol. Biotechnol.* 3, 207–220
21 Wilson, J. M. (1996) *New Engl. J. Med.* 334, 1185–1187
22 Yang, Y. and Wilson, J. M. (1995) *J. Immunol.* 155, 2564–2570
23 Yang, Y., Su, Q. and Wilson, J. M. (1996) *J. Virol.* 70, 7209–7212
24 Bramson, J., Hitt, M., Gallichan, W. S., Rosenthal, K. L., Gauldie, J. and Graham, F. L. (1996) *Hum. Gene Ther.* 7, 333–342
25 Gao, G. P., Yang, Y. and Wilson, J. M. (1996) *J. Virol.* 70, 8934–8943
26 Wang, Q. and Finer, M. H. (1996) *Nat. Med.* 2, 714–716
27 Wang, Q., Jia, X. C. and Finer, M. H. (1995) *Gene Ther.* 2, 775–783
28 Yeh, P., Dedieu, J. F., Orsini, C., Vigne, E., Denefle, P. and Perricaudet, M. (1996) *J. Virol.* 70, 559–565
29 Haecker, S. E. *et al.* (1996) *Hum. Gene Ther.* 7, 1907–1914
30 Parks, R. J., Chen, L., Anton, M., Sankar, U., Rudnicki, M. A. and Graham, F. L. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 13 565–13 570
31 Parks, R. J. and Graham, F. L. (1997) *J. Virol.* 71, 3293–3298
32 Miller, R. and Curiel, D. T. (1996) *Gene Ther.* 3, 557–559
33 Bischoff, J. R. (1996) *Science* 274, 373–376
34 Kirn, D. H. and McCormick, F. (1996) *Mol. Med. Today* 2, 519–527
35 Fink, D. J., Ramakrishnan, R., Marconi, P., Goins, W. F., Holland, T. C. and Glorioso, J. C. (1995) *Clin. Neurosci.* 3, 284–291
36 Marconi, P. *et al.* (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 11 319–11 320
37 Fink, D. J. and Glorioso, J. C. (1997) *Nat. Med.* 3, 357–359
38 Fink, D. J. and Glorioso, J. C. (1997) *Adv. Neurol.* 72, 149–156
39 Wu, N., Watkins, S. C., Schaffer, P. A. and DeLuca, N. A. (1996) *J. Virol.* 70, 6358–6369
40 Zhu, Z., DeLuca, N. A. and Schaffer, P. A. (1996) *J. Virol.* 70, 5346–5356
41 Starr, P. A. *et al.* (1996) *Gene Ther.* 3, 615–623
42 Neve, R. L. and Geller, A. I. (1995) *Clin. Neurosci.* 3, 262–267
43 Geller, A. I. (1997) *Adv. Neurol.* 72, 143–148
44 Rolling, F. and Samulski, R. J. (1995) *Mol. Biotechnol.* 3, 9–15
45 Samulski, R. J. (1993) *Curr. Opin. Genet. Dev.* 3, 74–80
46 Ferrari, F. K., Samulski, T., Shenk, T. and Samulski, R. J. (1996) *J. Virol.* 70, 3227–3234
47 Russell, D. W., Alexander, I. E. and Miller, A. D. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 5719–5723
48 Halbert, C. L., Alexander, I. E., Wolgamot, G. M. and Miller, A. D. (1995) *J. Virol.* 69, 1473–1479
49 Alexander, I. E., Russell, D. W. and Miller, A. D. (1994) *J. Virol.* 68, 8282–8287
50 Miller, A. D. (1997) *Nat. Med.* 3, 278–279
51 Fisher, K. J. *et al.* (1997) *Nat. Med.* 3, 306–312
52 Koeberl, D. D., Alexander, I. E., Halbert, C. L., Russell, D. W. and Miller, A. D. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 1426–1431
53 Kaplitt, M. G. *et al.* (1994) *Nat. Genet.* 8, 148–154
54 Fisher, K. J., Kelley, W. M., Burda, J. F. and Wilson, J. M. (1996) *Hum. Gene Ther.* 7, 2079–2087
55 Dubensky, T. W. *et al.* (1996) *J. Virol.* 70, 508–519

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DNA cancer vaccines: A gene gun approach

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Summary A wide variety of approaches, all using gene transfer, have been tested experimentally as alternative means to vaccinate against cancer, either prophylactically or therapeutically. These include both *ex vivo* and *in vivo* gene transfer to tumour and/or non-tumour cells, using both viral and non-viral vectors. The transferred DNA has varied widely as well, including genomic or cDNA encoding tumour-associated or oncofoetal antigens, cytokines, histocompatibility molecules, and costimulatory molecules. Several of these approaches have been applied in human clinical trials. This review summarizes those approaches, then compares and evaluates various methods using cytokine DNA in conjunction with autologous tumour cells, with particular emphasis on particle-mediated gene transfer via a gene gun. Finally, prospects and needs for further development are discussed.

Key words: cancer, DNA, gene therapy, vaccines.

Introduction

Recent advances in gene therapy research, nucleic acid vaccination methods, and cancer immunotherapy have provided opportunities for the application of these emerging technologies to the development of DNA vaccines for cancer. As recently reviewed by Roth and Cristiano,[1] Rosenberg[2] and others,[3–5] new approaches for cancer vaccines or cytokine-mediated cancer gene therapy have attracted many clinical investigators and molecular biologists in a concerted effort to explore this new research area. A number of quite distinct approaches, all using gene transfer, have been tested as alternative means to vaccinate against malignancies. These include: (i) the use of cDNA expression vectors encoding foreign class I or class II MHC antigen(s) for *ex vivo* or *in vivo* transfection of target tumour cells, with the aim of creating an immunostimulatory environment that might facilitate an immune response to not only the alloantigen-transfected tumour cells, but also to the non-transfected parental tumour;[6–8] (ii) the use of vectors expressing various cytokine transgenes (most notably granulocyte/macrophage colony-stimulating factor (GM-CSF)) to transfect *ex vivo* the weakly immunogenic animal tumours such as the B16 melanoma line, or human patients' autologous tumour cells, and then vaccinate the animals or cancer patients with the cytokine-expressing tumour cell vaccines, aiming to induce or augment the patient's immune response at the vaccine site, with the hope of obtaining a systemic antitumour response that extends to non-transfected, distant tumours;[5,9–12] (iii) the same approach as described in (ii), except that actively dividing fibroblasts from test animals or from human patients are employed as bioreactor cells for transgenic cytokine expression, and these cells are mixed and coinoculated with cells from tumour explants as DNA/cancer vaccine preparations;[13–15] (iv) the transgenic expression of CD80 (B7.1) costimulatory molecules in tumour cell vaccines for increased immune responses against target tumours;[11,15–17] (v) use of vectors encoding natural tumour-associated antigens (TAA; such as gp100, MART-1, MAGE antigens, tyrosinase) for DNA vaccination or breaking of tolerance against targeted melanoma or other cancers;[12,18] (vi) the use of gene(s) for oncofoetal antigens such as carcinoembryonic antigen for DNA vaccination against specific tumour types (e.g. colon cancer);[19] and (vii) potentially a combination of one or more of the above DNA vaccination methods in a 'cocktail' approach.[5,11,12,14,16]

In addition to the increasing number of potential DNA cancer vaccine strategies, as already described, a variety of means and tools have been evaluated for the gene delivery itself. Using primarily mouse model systems, most gene therapy investigations to date have employed virus-mediated gene transfer systems,[1,3,18] including replication-deficient retrovirus, adenovirus, adeno-associated virus and vaccinia virus, to deliver usually one or two transgenes. Non-viral gene delivery methods have also been developed, in the hope of achieving improved safety or reduced immunoreactivity compared to viral vectors.[1,3,4,8,20] A number of non-viral vector systems, including liposomes, naked DNA and particle- or gene gun-mediated DNA delivery have also been employed, modified and improved for *ex vivo* or *in vivo* gene transfer of DNA vaccines into target tumours, or into admixed or adjacent normal cells or tissues. In this short review, because of our own research experience and our projection for an increased future application of the technology, we have chosen to focus on the specific example of gene gun-mediated transfection of GM-CSF cDNA into target tumour cells. Around this focus, we will address the various important and emerging issues related to DNA cancer vaccines, including the efficiency and pattern of transgene expression, the degree of efficacy in animal models, and the prospects and potential pitfalls for application to human clinical trials.

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Cytokine gene-transfected tumour cells as cancer vaccines

Stimulation of a patient's own immune effector cells to kill autologous tumour has been the elusive target of a number of studies of cytokine therapy in patients with cancer. For this strategy to effectively eradicate an established tumour, that may or may not have metastasized, a tumour-specific immune response must be generated where no response, or even a tumour-enhancing response, existed previously. The assumption of most cytokine gene or cytokine protein therapy trials is that the response of immune effector cells can be greatly stimulated or augmented by exogenously or transgenically administered cytokines, resulting in activation of specific T cells or non-specific, inflammatory cell activities. Either or both of these cellular immune activities are hoped to be sufficient to mediate a potent antitumour response. Stimulation of the immune system by exogenous cytokines has effectively eradicated certain immunogenic tumours in rodent models.[21] However, translation of these animal studies to human cancer clinical trials has been much less promising.[22,23] The most successful human studies in patients with non-resectable tumours have focused on treatment with IL-2, IL-2 plus *ex vivo*-stimulated autologous lymphocytes, or IL-2 plus other cytokines such as IFN-α or GM-CSF.[23–27] In most cases, the best responses were obtained in patients receiving high cytokine doses.[23] Further escalation of the cytokine dose was most often precluded by the associated systemic toxicity.[28] High but localized cytokine doses, administered in the vicinity of tumour cells, were then contemplated as an alternative means to generate a systemic immune response. Such an approach, to achieve high local cytokine levels without systemic toxicity, has been the object of intensive experimental investigations. A number of *ex vivo* and *in vivo* gene therapy strategies provide a possibility for this approach.

A number of animal studies have shown that vaccination with tumour cells transfected with specific cytokine gene expression vectors can protect animals from subsequent challenge with wild-type tumours.[1–11,29,30] Dranoff *et al.*,[9] using retrovirally transduced tumour cells in the poorly immunogenic B16 mouse melanoma model, injected those cells *in vivo* and achieved a vaccinating effect against parental B16 cells. The greatest protective effect was achieved with GM-CSF expression and required cytokine production above a given threshold level.[9] We have recently confirmed this dosage observation in the same B16 melanoma model, using a gene gun for gene transfer to the tumour cells, and further demonstrated that genetic vaccination with cells expressing GM-CSF was more effective than similar treatment with cells expressing IL-2, IFN-γ, or IL-12.[10,31]

Several clinical gene therapy protocols are likewise based on delivery of cytokine DNA to clonal tumour cell populations.[32] This approach was used because retroviral vectors can only effectively infect replicating cells.[33] General application of the retroviral approach is limited by the fact that after tumour resection, often only a very small population of tumour cells may be dividing or capable of being induced to proliferate in culture. Furthermore, the cell culture periods employed by most tumour vaccination protocols may cause a shift or reduction in the spectrum of cellular phenotypes and potential tumour antigens present. Thus, a gene transfer technology that can be effective immediately following tumour biopsy or resection, and can yield sufficient production of transgenic immunomodulatory proteins, may provide a highly desirable cancer vaccine approach.

GM-CSF-transfected autologous tumour cells as cancer vaccines: Rationale and limitations

Vaccination of animals with irradiated tumour cells alone has been shown to prevent growth of subsequent tumour in immunogenic murine tumour models, but had minimal prophylactic effect in non-immunogenic tumour models such as the B16 melanoma. We have confirmed in animal studies that vaccination with tumour cells transfected with cytokine genes can protect animals from subsequent challenge with wild-type tumour.[10] Specifically, particle-mediated transfection of the B16 mouse melanoma cell line, with the murine GM-CSF-expressing plasmid vector pWRG-3142, resulted in high-level expression of the transgenic mGM-CSF protein in the tissue culture supernatant that was sustained for 10 days *in vitro*. When 1×10^6 gamma-irradiated mGM-CSF-transfected B16 cells were implanted as a s.c. vaccine, 58% of test mice were protected from subsequent challenge with 1×10^5 non-irradiated, non-transfected parental tumour cells. In contrast, only 2% of mice were protected from the same challenge when the vaccinating B16 cells had been transfected with control (luciferase) DNA (Fig. 1). Furthermore, the tissue space surrounding the mGM-CSF-transfected, but not the control-transfected, tumour vaccine was dominated by an inflammatory infiltrate. Both the antitumour effect and the histological results confirm that transgenic expression of GM-CSF results in functional activity in vaccinated mice.

Similar results from a number of laboratories have led to many proposed human gene therapy trials based on *ex vivo* transfer of GM-CSF expression vectors into tumour cells (see reference 1). The key differences among the various clinical trials are related to the method of gene transfer into the autologous tumour cells and to the tumour type targeted for study. It is essential to consider the design of the preclinical studies that served as an impetus to these clinical trials. A major concern about these protective preclinical experiments is the lack of a model that demonstrates the killing of an established tumour. The current protection models may translate to adjuvant therapy trials but may not predict antitumour effects in patients with non-resectable established tumour(s) (a typical patient in phase II trials), and thus the putative efficacy may not be demonstrated in clinical trials.

All preclinical models and human trials require appropriate expression of the candidate therapeutic cytokine transgene(s). As a great majority of clinical tumour cell vaccine studies are designed to use irradiated tumour cells and as, with the gene gun or other non-viral techniques, the cytokine gene is rarely integrated into the tumour DNA, genetic safety concerns with this approach are minimal. Thus, such *ex vivo* gene transfer, cancer vaccine approaches are considered to have met the established, very stringent genetic safety standard.

The potential transgenic expression of GM-CSF *in situ* can be estimated from the expression level detected in the same cells in culture or, alternatively, from the level of transgenic protein detected in biopsy samples from tissue sites implanted with transfected tumour cells. Each of these approaches

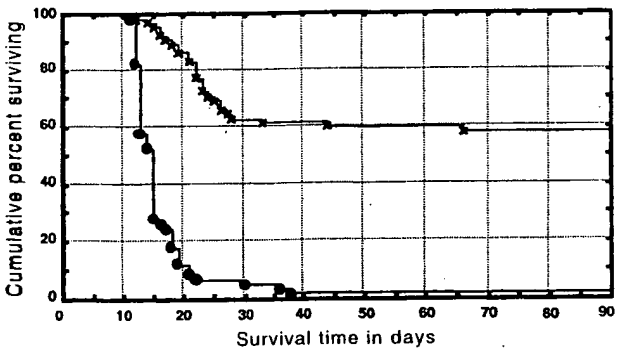

Figure 1 Survival time of immunized mice. C57B1/6 mice were immunized with 1×10^6 γ-irradiated murine B16 melanoma cells transfected with (X) mGM-CSF or (●) luciferase cDNA in identical plasmid expression vectors. Seven days after vaccination, each animal was challenged on the contralateral abdominal site with 1×10^5 live parental B16 tumour cells. Tumours present were measured by taking two perpendicular measurements with calipers. When the tumour area exceeded 100 mm^2, the mouse was killed, and the interval between challenge and sacrifice was used as the individual survival time. All surviving animals were followed for at least 90 days. Results are cumulative data from eight independent experiments involving a total of 122 animals (65 receiving GM-CSF-transfected tumour cells, and 57 receiving luciferase-transfected tumour cells as the vaccine). No differences were noted among the eight experiments, for either the mGM-CSF-transfected vaccine groups (Chi-squared, 7 d.f. = 8.4) or the control-transfected vaccine groups (Chi-squared, 7 d.f. = 7.4). The difference between the mGM-CSF and control groups was statistically significant (Chi-squared, 1 d.f. = 93.4; $P = 0.0001$). Reproduced from *Hum. Gene Ther.* 1996; 7: 1535–43 by copyright permission of Mary Ann Liebert, Inc. Publishers.

has its advantages and disadvantages. A dose–response effect has been noted in murine models by Dranoff *et al.*[9] and confirmed by our group.[34] The generation of local GM-CSF concentrations can be as low as 10 ng/10^6 cells per 24 h and still be effective. This seems to be largely independent of the number of tumour vaccine cells; thus, the local cytokine concentration can be enhanced by increasing the number of DNA-treated tumour cells injected at the vaccine site. Translation of these murine data to humans requires the assumption that local cytokine/tumour/effector cell interactions are not only similar in rodents and primates, but basically independent of animal size. This may be a reasonable assumption but remains unproven. A number of methods of cytokine gene transfer into the autologous tumour cells have been proposed, including the use of retrovirus, adenovirus, liposomes, and microscopic gold particles as vectors for gene transfer. Three methods are now under investigation in various approved clinical trials, as detailed below.

Techniques in current clinical trials for cytokine gene transfer to autologous tumour cells

For cancer gene therapy studies, retroviral insertion of GM-CSF, IL-2, IL-12 or IFN-γ DNA has been the focus of most clinical trials to date. Among these, GM-CSF is the most intensively studied. All retrovirus-mediated gene transfer trials require culture of rapidly dividing, autologous tumour cells which can be effectively infected with a non-replicating retrovirus. When an adequate number of transduced cells are generated, an irradiated intradermal tumour vaccine is placed. This protocol is designed to result in stable transgenic GM-CSF expression at a sufficient level, in a relatively homogeneous tumour cell population, and is without significant safety concerns. The apparent negatives are: (i) the short-term expression inherent in all tumour cell vaccine strategies (the tumour vaccine cells will soon be killed if the vaccine is effective); (ii) the need for prolonged cultivation, often lasting 1–3 months, which may limit the routine application of this procedure for reasons of time and treatment cost; and (iii) the selected, mitotically active population of tumour cells derived from enrichment in culture, which may select an antigenically limited subpopulation from the original tumour cell explant.

Adenoviral and adeno-associated viral vectors have also been employed for DNA delivery in cancer vaccine research and clinical trials. Adenovirus (AV) apparently can transduce non-dividing cells, and high-level transient expression is a unique feature of the AV vectors. Immunity against viral proteins of AV vectors at high multiplicity of infection may be problematic for certain clinical cancer vaccine approaches. Adeno-associated virus (AAV) is valued for its site-specific integration of transgenes into genomic DNA of the target cells. Adeno-associated virus has a very limited genome and transgene loading capacity, which can be a disadvantage.

A third class of technique for tumour cell transfection is the delivery of naked DNA without a viral vector. Several methods to avoid the need for tissue/cell culture manipulation have been evaluated, including direct transfer of DNA into tumour *in situ*, and immediate transfection of freshly explanted tumour cells. For direct gene transfer, DNA can traverse the cell membrane by physical means such as a gene gun (or particle-mediated gene transfer; PMGT) or by active uptake by the cells (as DNA alone or as DNA–liposome complexes). The PMGT method is now the focus of a clinical trial. This technology may have some advantages over virus-mediated gene transfer methods. Because the DNA is physically propelled into the targeted cell, active uptake (as in endocytosis of liposomes) or infection is not required. This feature allows transfection of non-dividing cells and avoids the need for specific receptors or cell functions that may be limited to particular cell types. Cell culture manipulation is not necessary, and this may result in significant cost savings relative to virus-mediated gene transfer. Potential disadvantages of PMGT include the relatively short period (7–10 days) of transgene expression, and the lack of a homogeneous, dividing tumour cell population for administration as a vaccine. However, a homogeneous, mitotically active tumour cell population may, in fact, not be a desirable feature as the original, heterogeneous tumour explant cell population may present more antigens to the patient's immune system. The schema for a gene gun-mediated, human cancer vaccine trial currently accruing patients is outlined in Fig. 2. All steps from tumour removal to implantation of DNA-transfected tumour cells can be accomplished in less than 6 h.

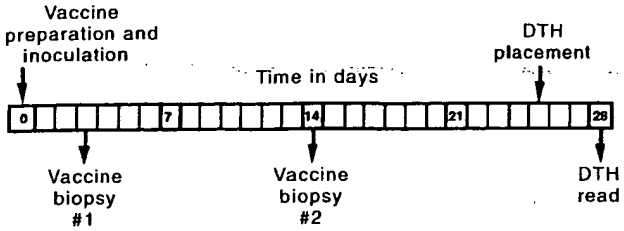

Figure 2 Schema for clinical treatment protocol in a gene gun-mediated cancer DNA vaccine trial, in which granulocyte/macrophage colony-stimulating factor (GM-CSF) cDNA is transferred into autologous tumour cells *ex vivo*. This ongoing phase I trial addresses the toxicity of two dose levels of GM-CSF DNA-transfected tumour cell vaccines. The dose is controlled by varying the per cent of transfected cells in the gamma-irradiated tumour cell vaccine. Group 1 patients receive an immunization in which 10% of the cells are exposed to transfection with the GM-CSF gene and 90% are not transfected. This cell mixture is placed intradermally in two separate sites as tumour vaccines. The number of tumour cells is held constant between groups. Group 2 patients receive an immunization in which 100% of the tumour cells have been exposed to particle-mediated gene transfer of GM-CSF. The second group will receive GM-CSF doses comparable to a biologically effective dose as defined in murine trials.

Future prospects

Gene transfer technologies

A majority of the research related to gene therapy has necessarily focused on technological development of gene delivery systems. The rapid proliferation of experimental and clinical gene therapy studies has also been driven by the increasing variety of vectors for gene delivery. Fortunately, many different viral and non-viral gene delivery systems have proven in general to be safe, reliable and efficacious. *Ex vivo* methods such as the gene gun technique, that bypass the need for cell culture, have allowed the targeting of (a) non-dividing cells, (b) multiple cell types (including skin dendritic cells, for example) that may better present tumour antigens to the immune system, and (c) solid tumours accessible surgically. The treatment of malignant conditions such as leukaemia, that are not readily amenable to current means of gene transfer, will require a targeting system that specifically targets the malignant cells and then inserts the gene of interest, all within the circulation.

Candidate therapeutic genes

Although current trials have focused largely on GM-CSF, plus a few studies of interleukins (IL-2 and IL-12) and interferons, new discoveries will likely soon change our understanding of various immunological phenomena and lead to therapies aimed at different means to enhance the immune response to autologous tumours. The explosive advancement in the molecular biology of the immune response has expedited the (sometimes monthly) identification of genes encoding new and promising cytokines, chemokines and immune costimulatory or modifier signals.

The presently available genes, and those still to be discovered, may not function optimally as a single drug. DNA vaccine cocktails encoding combinations among cytokines, histocompatibility (HLA) molecules, costimulatory molecules such as CD80 (B7.1), and tumour-associated antigens such as gp100 and MART-1, may prove to be more effective than singular treatment. Similarly, combinations of therapeutic modalities, such as gene therapy plus systemic cytokine protein therapy, may be superior to any of these approaches alone.

Target tissue/cell type for transfection

In most studies to date, the target tissue for gene transfer has been syngeneic or autologous tumour. Recent studies suggest, however, that professional APC such as dendritic cells and macrophages may be superior immune stimulants. This may lead to many interesting trials with antigen-encoding DNA for tumours such as melanoma, that have defined cell surface antigens, but may have reduced relevance in other common malignancies unless new tumour-specific antigens are identified.

We have previously demonstrated the capacity of gene gun-mediated DNA transfer to achieve transgenic expression in a wide range of freshly excised tissues, cell explants, cells collected from body fluids, biochemically processed cell aggregates or tissue clumps, and primary cell cultures derived from these tissue sources (see review in reference 4). Thus, there is a high probability that this purely physical means for gene transfer may provide an efficient and convenient gene transfer system for generic application to clinical tumour cell samples, for the production of DNA-transfected cancer cell vaccines. This projection is supported by a recent tumour vaccine study comparing the efficacy and efficiency of retroviral and gene gun methods for *ex vivo* gene transfer to tumour cells (T Tüting and MT Lotze, pers. comm. 1997).

Establishing clinical relevance

To date, the clinical relevance of biological therapy for cancer has only been established in patients with high-risk, but completely resected melanoma. The efficacy of gene-based biological therapy must not solely be confined to phase I trials with various biological end-points, no matter how scientifically interesting, but must be extended to well-designed phase II and III clinical trials. Only then will this technology have demonstrated its clinical relevance in human disease.

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References

1 Roth JA, Cristiano RJ. Gene therapy for cancer: What have we done and where are we going? *J. Natl Cancer Inst.* 1997; **89**: 21–39.

2 Rosenberg SA. Development of cancer immunotherapies based on identification of the genes encoding cancer regression antigens. *J. Natl Cancer Inst.* 1996; **88**: 1635–44.
3 Gilboa E. Immunotherapy of cancer with genetically modified tumor vaccines. *Semin. Oncol.* 1996; **23**: 101–7.
4 Yang NS, Sun WH, McCabe D. Developing particle-mediated gene-transfer technology for research into gene therapy of cancer. *Mol. Med. Today* 1996; **2**: 476–81.
5 Dilloo D, Bacon K, Holden W *et al.* Combined chemokine and cytokine gene transfer enhances antitumor immunity. *Nat. Med.* 1996; **2**: 1090–5.
6 Ostrand-Rosenberg S, Clements VK, Thakur A, Cole GA. Transfection of major histocompatibility complex class I and class II genes causes tumor rejection. *J. Immunogenet.* 1989; **16**: 343–9.
7 Armstrong TD, Clements VK, Martin BK, Ting JPY, Ostrand-Rosenberg S. Major histocompatibility complex class II-transfected tumor cells present endogenous antigen and are potent inducers of tumor-specific immunity. *Proc. Natl Acad. Sci. USA* 1997; **94**: 6886–91.
8 Nabel GJ, Nabel EG, Yang ZY *et al.* Direct gene transfer with DNA–liposome complexes in melanoma: Expression, biological activity, and lack of toxicity in humans. *Proc. Natl Acad. Sci. USA* 1993; **90**: 11 307–11.
9 Dranoff G, Jaffee E, Lazenby A *et al.* Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific and long-lasting anti-tumor immunity. *Proc. Natl Acad. Sci. USA* 1993; **90**: 3539–43.
10 Mahvi DM, Burkholder JK, Turner J *et al.* Particle-mediated gene transfer of granulocyte-macrophage colony-stimulating factor cDNA to tumor cells: Implications for a clinically relevant tumor vaccine. *Hum. Gene Ther.* 1996; **7**: 1535–43.
11 Aruga E, Aruga A, Arca MJ *et al.* Immune responsiveness to a murine mammary carcinoma modified to express B7-1, interleukin-12 or GM-CSF. *Cancer Gene Ther.* 1997; **4**: 157–66.
12 Irvine KR, Rao JB, Rosenberg SA, Restifo NP. Cytokine enhancement of DNA immunization leads to effective treatment of established pulmonary metastases. *J. Immunol.* 1996; 156: 238–45.
13 Zitvogel L, Tahara H, Robbins PD *et al.* Cancer immunotherapy of established tumors with IL-12: Effective delivery by genetically engineered fibroblasts. *J. Immunol.* 1995; **155**: 1393–403.
14 Tahara H, Lotze MT, Robbins PD, Storkus WJ, Zitvogel L. IL-12 gene therapy using direct injection of tumors with genetically engineered autologous fibroblasts. *Hum. Gene Ther.* 1995; **6**: 1607–24.
15 Townsend SE, Allison JP. Tumor rejection after direct co-stimulation of $CD8^+$ T cells by B7-transfected melanoma cells. *Science* 1993; **259**: 368–70.
16 Albertini MA, Emler CA, Schell K, Tans KJ, King DM, Sheehy MJ. Dual expression of human leukocyte antigen molecules and the B7-1 costimulatory molecule (CD80) on human melanoma cells after particle-mediated gene transfer. *Cancer Gene Ther.* 1996: 3: 192–201.
17 Chen L, Ashe S, Brady WA *et al.* Co-stimulation of antitumour immunity by the B7 counter-receptor for the T lymphocyte molecule CD28 and CTLA-4. *Cell* 1992; **71**: 1093–102.
18 Zhai Y, Yang JC, Kawakami Y *et al.* Antigen-specific tumor vaccines: Development and characterization of recombinant adenoviruses encoding MART1 or gp100 for cancer therapy. *J. Immunol.* 1996; **156**: 700–10.
19 Conry RM, Widera G, Lobuglio AF *et al.* Selected strategies to augment polynucleotide immunization. *Gene Ther.* 1996; **3**: 67–74.
20 Yang N-S, Sun WH. Gene gun and other non-viral approaches for cancer gene therapy. *Nat. Med.* 1995; **1**: 481–3.
21 Mule JJ, Yang JC, Lafreniere RL, Shu S, Rosenberg SA. Identification of cellular mechanisms operational *in vivo* during the regression of established pulmonary metastases by the systemic administration of high dose recombinant interleukin-2. *J. Immunol.* 1987; **139**: 285–94.
22 Borden EC, Sondel PM. Lymphokines and cytokines as cancer treatment: Immunotherapy realized. *Cancer* 1990; **65**: 800–14.
23 Rosenberg SA, Yang JC, Topalian SL *et al.* Treatment of 283 consecutive patients with metastatic melanoma or renal cell cancer using high dose bolus interleukin-2. *J. Am. Med. Assoc.* 1994; **271**: 907–13.
24 Sosman JA, Kohler PC, Hank JA, Moore KH, Bechhofer R, Storer B, Sondel PM. Repetitive weekly cycles of recombinant human interleukin-2: Responses of renal carcinoma with acceptable toxicity. *J. Natl Cancer Inst.* 1988; **80**: 60–3.
25 Thompson JA, Shulman KL, Benyunes MC *et al.* Prolonged continuous intravenous infusion interleukin-2 and lymphokine-activated killer cell therapy for metastatic renal cell carcinoma. *J. Clin. Oncol.* 1993; **10**: 960–8.
26 Schiller JH, Hank JA, Storer B *et al.* A direct comparison of immunological and clinical effects of interleukin-2 with and without interferon-α in humans. *Cancer Res.* 1993; **53**: 1286–92.
27 Hank JA, Albertini M, Wesly OH *et al.* Clinical and immunologic effects of treatment with murine anti-CD3 monoclonal antibody together with interleukin-2 in patients with cancer. *Clin. Cancer Res.* 1995; **1**: 481–91.
28 Parkinson DR. Lessons from the clinical trials of interleukin-2. *Natl Immunol. Cell Growth Regul.* 1990; **9**: 242–52.
29 Cavallo F, Dipierro F, Giovarelli M *et al.* Protective and curative potential of vaccination with interleukin-2 gene-transfected cells from a spontaneous mouse mammary adenocarcinoma. *Cancer Res.* 1993; **53**: 5067–70.
30 Schmidt W, Schweighoffer T, Herbst E *et al.* Cancer vaccines: the interleukin-2 dosage effect. *Proc. Natl Acad. Sci. USA* 1995; **92**: 4711–14.
31 Sun WH, Burkholder JK, Sun J *et al. In vivo* cytokine gene transfer by gene gun suppresses tumor growth in mice. *Proc. Natl Acad. Sci. USA* 1995; **92**: 2889–93.
32 Berns AJ, Clift S, Cohen LK *et al.* Phase I study of non-replicating autologous tumor cell injections using cells prepared with or without GM-CSF gene transduction in patients with metastatic renal cell carcinoma. *Hum. Gene Ther.* 1995; **6**: 347–68.
33 Miller DG, Adam MA, Miller AD. Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. *Mol. Cell. Biol.* 1990; **10**: 4239–42.
34 Mahvi DM, Sondel PM, Yang N-S *et al.* Phase I/IB study of immunization with autologous tumor cells transfected with the GM-CSF gene by particle-mediated transfer in patients with melanoma or sarcoma. *Hum. Gene Ther.* 1997; **8**: 875–91.

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Osteoprogenitor Cells as Targets for Ex Vivo Gene Transfer

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ABSTRACT

We transduced osteoprogenitor cells with recombinant retrovirus and analyzed proviral integration patterns into chromosomal DNA to detect for the first time the clonal and cellular fate of osteoprogenitor-derived progeny cells. Metaphyseal bone cells and diaphyseal stromal cells were isolated from the distal femurs of young rats, transduced with the vM5*neolacZ* recombinant retrovirus, and selected in the neomycin analog, G418. Following surgical marrow ablation of a femur in one leg of mature rats, retroviral-transduced metaphyseal or diaphyseal cells were injected into the ablated site. These rats were killed 5-6 days later. Metaphyseal and diaphyseal cells were isolated from distal femurs, selected in G418, and stained for β-galactosidase (β-gal+). The number and clonal origin of transduced progenitor cells were determined. High numbers of β-galactosidase colonies with an osteoblast phenotype were obtained following metaphyseal transplants and detected in 100% of metaphyseal and none of diaphyseal specimens. In contrast, β-galactosidase colonies derived from diaphyseal transplants were detected in 50% of specimens in both the metaphysis and diaphysis, and the absolute number of progenitor cell colonies was 60-fold less than metaphyseal transplants. Provirus was only detected in the ablated bones and not in the contralateral bone or other tissues. Proviral integration fragment analysis showed a single integration site for recovered metaphyseal cell clones, consistent with their origination from a common single progenitor. This is one of the first demonstrations of successful transplantation of clonal osteoprogenitors to their site of origin in bone. It may be possible to use these cells to target genes to bone for therapeutic use in skeletal and hematopoietic diseases. (J Bone Miner Res 1998;13:20–30)

INTRODUCTION

The process of bone formation involves the continual generation of newly formed osteoblasts, which generate new bone as they proceed to terminal differentiation. This continual supply of osteoblasts is sustained by a supporting population of mesenchymal stem cells and intermediate osteoprogenitor cells.[1,2] The precise location of these repositories of mesenchymal stem and osteoprogenitor cells, as well as the interval between the onset of stem and progenitor cell proliferation to differentiated bone cells, is not well defined. In postnatal bone, repositories of mesenchymal stem cells may lie within the marrow, periosteum, and muscle connective tissue.[3-9] For example, cells from these sites differentiate into bone when transplanted into ectopic sites in vivo, or when cultured in vitro in the presence of supplements such as vitamin C, β-glycerolphosphate, and dexamethasone.[3,6,7,9-12] However, the exact role and contribution of cells from these sites to normal bone homeostasis in vivo is not clear.

In long bones of young rats, using [^{3}H]thymidine or bromodeoxyuridine uptake, we and others have demonstrated that, within the primary spongiosa, in the transition zone between hypertrophic chondrocytes and trabecular bone, proliferating osteoprogenitor cells are predominantly localized to the region subjacent to the growth plates and cortical endosteum.[13-15] Proliferative cells subjacent to the growth plates provide the osteoprogenitors for trabec-

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ular bone of the metaphyseal primary spongiosa, while those subjacent to the endosteum contain mostly the precursors for hematopoietic cells and the various subpopulations of stromal cells, including diaphyseal endosteal osteoblasts. We also reported that parathyroid hormone (PTH) targets the proliferating osteoprogenitors in primary spongiosa to increase the number differentiating into functional osteoblasts.[16,17]

We recently isolated the primary spongiosa cells of young rats for in vitro studies.[15,18] These cells retained their ability to proliferate and differentiated to osteoblasts. We hypothesized that these isolated primary spongiosa cells included a small subset of cells that would be progenitor in nature. We investigated whether these cells from young, rapidly growing rats could successfully compete with endogenous repopulating proliferating osteoprogenitors when reimplanted into an ablated femur of an adult rat. The aim of the present study was to develop a somatic gene transfer technique to infect proliferating bone cells, ex vivo, with a replication-defective retrovirus encoding the neomycin resistance gene, *neo,* and the histochemical reporter gene, *lacZ,* and then to map the fate of these cells when reimplanted in vivo.

Within a week of implantation, hundreds of β-galactosidase positive colonies of primary spongiosa cells were recovered only from metaphyseal sites. In contrast, in diaphyseal sites, the number of β-galactosidase positive colonies recovered was 60-fold less, and these were detectable in only ~50% of the sites. Analysis by polymerase chain reaction (PCR) showed the provirus to be present only in the ablated bone and not in any other organ or the contralateral leg bones. Southern analyses showed a single integration site for recovered primary spongiosa cells, suggesting the proliferation of a single parent progenitor. Our results show successful selective transplantation of clonal osteoprogenitors to their site of origin in bone and also support the hypothesis that, like stem and progenitor cells in the hematopoietic system, a low number of primitive proliferating cells can generate a clonally expanded population of committed osteoprogenitors.

MATERIALS AND METHODS

Chemicals and reagents

Tissue culture reagents were obtained from GIBCO BRL (Grand Island, NY, U.S.A.). Fetal bovine serum (FBS) was from Hyclone Laboratories (Logan, UT, U.S.A.). Geneticin (G418) was obtained from Sigma (St. Louis, MO, U.S.A.), and [α-^{32}P]dCTP was purchased from New England Nuclear Corp. (Boston, MA, U.S.A.). Taq polymerase and the reagents for DNA amplification were purchased from Perkin Elmer Cetus (Norwalk, CT, U.S.A.) and used as recommended by the manufacturer. All other molecular biology reagents were obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.) and used as suggested by the manufacturer. General laboratory chemicals were from Sigma.

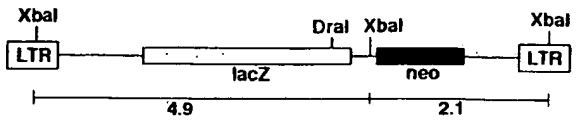

FIG. 1. Structure of the retroviral vector pM5*neolacZ*. The parent vector pM5*neo* was obtained from C. Stocking.[48] A 3.7 kb *Bam*HI *lacZ* fragment was *Bam*HI adapted and ligated into the unique *Bam*HI site, B, in the pM5*neo* retroviral backbone. The *neo* gene previously cloned into the backbone 3′ to splice acceptor sequences renders cells resistant to the neomycin analog G418. Both genes are transcribed from the 5′ LTR. D, *Dra*I restriction endonuclease cleaves the provirus only once and allows for the creation of junctional fragments containing proviral and genomic DNA.

Animals

Young and mature virus-antibody-free male Sprague-Dawley rats, 70-100 g and 245-255 g, respectively, were purchased from Harlan Laboratories (Indianapolis, IN, U.S.A.) and fed Purina rat chow (calcium 1%, phosphate 0.61%; PMI Feeds, Inc., St. Louis, MO, U.S.A.) and water ad libitum. The young rats were used as the source of metaphyseal and diaphyseal osteoprogenitor cells. The mature rats were used as hosts for cell transplantation. Animal protocols were approved by the Lilly Animal Care Committee.

Retroviral vector

The vM5*neolacZ* retrovirus contains the cDNA of the neomycin resistance gene *neo* and the histochemical reporter gene *lacZ* and has been previously described (Fig. 1). There is a unique *Dra*I restriction endonuclease site within the *lacZ* cDNA of the provirus. *Dra*I digestion of genomic DNA encoding the provirus allows for the creation of unique DNA integration fragments that contain both proviral and flanking genomic DNA sequences. The length of the flanking genomic DNA identified in the fragments is variable and dependent on the location of the most proximal *Dra*I site in genomic DNA adjacent to the provirus. Hybridization of DNA with a radiolabeled *Bam*HI–*Cla*I fragment of *lacZ* as a probe allows detection of the 5′ region of the provirus and genomic DNA flanking the provirus. Hybridization of DNA with the *neo* probe allows detection of the 3′region of the provirus and DNA 3′ to the proviral integration site. The retrovirus plasmid was packaged in the GP+E86 packaging line, and the clone used for all experiments had a titer of 1×10^6 G418r cfu/ml on NIH3T3 cells and was determined to be free of replication-competent virus.[19,20] Virus production, titering of recombinant supernatant, and evaluation for the existence of replication-competent virus from packaging cells and animals were performed as previously described.[19,21]

Harvest and cultivation of metaphyseal and diaphyseal marrow osteoprogenitors

Metaphyseal and diaphyseal osteoprogenitor cells were obtained from the distal femur of young rats as previously

described.[15,18] Cells obtained using this procedure are 80–90% morphologically homogenous, rapidly differentiate, and display osteoblastic characteristics such as high alkaline phosphatase (ALP) expression, cyclic adenosine monophosphate (cAMP) accumulation in response to PTH, the ability to synthesize predominantly type I collagen, and the ability to retain the capacity to form calcified bone-like tissue in vitro.[15] Following euthanasia, femurs were resected, and all exterior connective tissue, including periosteum, was completely removed and bone cell cultures prepared as described.[15,18]

Metaphyseal bone cultures

The distal epiphysis was removed, and a subjacent 2–3 mm band of the primary spongiosa was resected (metaphyseal section), minced, and digested for 1 h at 37°C in trypsin (Sigma). After neutralization of trypsin with trypsin inhibitor (Sigma), cells were filtered through a 70 μm pore cell strainer (Becton Dickinson and Co., Lincoln Park, NJ, U.S.A.) and counted. Cells were plated at 1–2 $\times$ 10^6 cells/100-mm plate in alpha-modified essential medium (α-MEM; GIBCO BRL), with 20% FBS and 1% penicillin-streptomycin (GIBCO BRL), and maintained at 37°C in 95% humidity with 5% CO_2. The medium was replaced every 3–4 days, with α-MEM containing 10% FBS and 1% penicillin-streptomycin.

Diaphyseal marrow culture

The diaphyseal middle third of the same femurs was cut out and the marrow flushed with fresh α-MEM containing 10% FBS and 1% penicillin-streptomycin, and prepared as single-cell suspensions. Cells were counted and viable cells plated at 1–2 $\times$ 10^7 cells/100-mm plate and maintained at 37°C in 95% humidity with 5% CO_2. As described by Owen et al.,[22] cells were allowed to adhere in the absence of media changes for 6 days, and then media was changed every 3–4 days.

Alkaline phosphatase activity

After colonies or aggregates were established, medium was replaced by fixative and cells processed for histochemical staining with ALP, using sigma kit 245 (Sigma). In confluent cell cultures, cell-associated ALP activity was quantitated by a colorimetric method as previously described[23] and expressed in enzyme units as micromoles of p-nitrophenol phosphate (PNP) cleaved per hour per milligram of protein (data not shown).

Transduction with retroviral vectors

Primary cultures of metaphyseal and diaphyseal osteoprogenitor cells were subcultured at 5 $\times$ 10^5 cells/100-mm plate 1 day prior to infection. Retroviral transduction was performed by incubating cells in 1 ml of recombinant supernatants containing virus (1 $\times$ 10^6/ml), 3 ml of cell culture media containing 10% FBS, and polybrene (final concentration 8 μg/ml). After 3 h, fresh supernatants containing virus and culture media were added, and incubation was continued overnight. Incubation with virus was repeated the next day as described. Control primary cultures of metaphyseal and diaphyseal osteoprogenitor cells were incubated

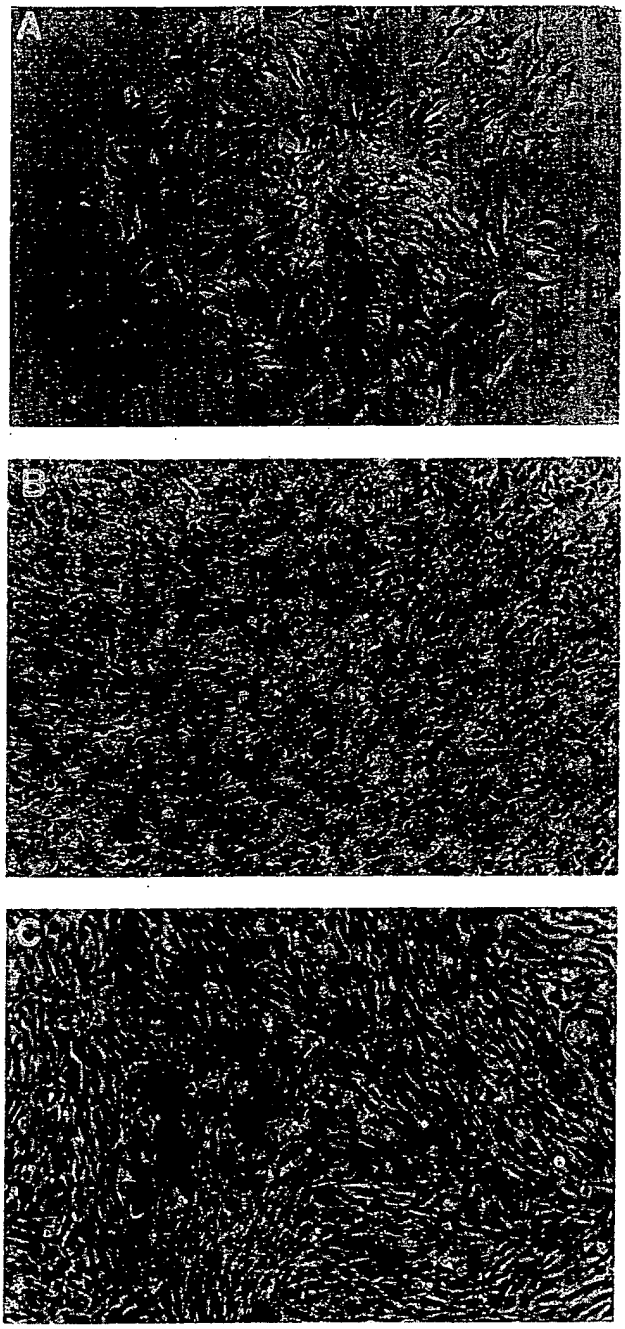

FIG. 2. (A) Phase contrast photomicrograph showing aggregation of osteoprogenitor cells from the metaphyseal primary spongiosa of a distal femur 12 h after isolation. The small round cells are mononuclear hematopoietic (marrow) cells that disappear when medium was changed. (B) Phase contrast microscopy showing confluent culture of cells derived from the primary spongiosa in 7 days. (C) Confluent colony of diaphyseal marrow stromal cells obtained from the same animal as the primary spongiosa cells.

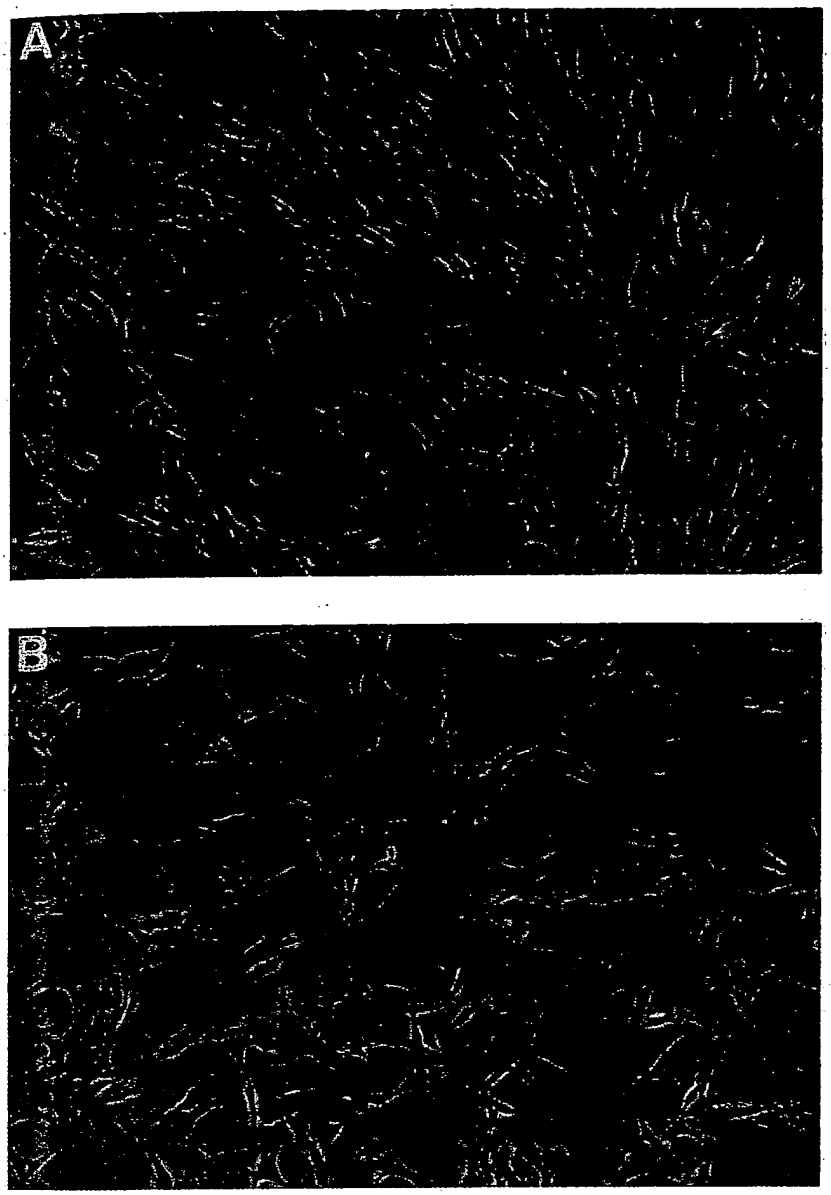

FIG. 3. X-gal staining of (A) metaphyseal derived primary spongiosa cells and (B) diaphyseal derived stromal cells infected with vM5*neolacZ* retrovirus expressing the histochemical reporter gene *lacZ*. Primary cultures of metaphyseal and diaphyseal osteoprogenitor cells were subcultured and transduced with supernatants containing retrovirus as described in the Materials and Methods section. Selection in G418 was continued for 12 days with regular media changes every 3–4 days. G418-resistant colonies were stained for β-galactosidase. The blue color represent cells expressing the *lacZ* gene.

in media alone (without virus). On the third day, cultures were either stained for β-galactosidase or split 1:10 into culture medium containing 1 mg/ml G418. Selection in G418 was continued for 12 days with regular media changes every 3–4 days. G418-resistant colonies were stained for either β-galactosidase and/or ALP.

β-galactosidase histochemistry

We used a histochemical stain for β-galactosidase[24] to detect expression of *lacZ* in virus-infected cells. Cultured cells were rinsed with PBS and fixed for 5 minutes at 4°C with 0.2% glutaraldehyde-2% formaldehyde in PBS. The cells were rinsed with PBS and then incubated at 37°C overnight in staining solution containing 1 mg/ml X-Gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM magnesium chloride in PBS as previously described.[19,24,25] For whole organ staining, bone histomorphometry was done using conventional techniques on undecalcified frozen sections of the distal femur. X-Gal staining was done as described[24] except that the tissue specimens were preincubated in staining buffer containing 0.3 mM chloroquine, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM magnesium chloride before the addition of 1 mg/ml X-Gal.

Transplantation and recovery of bone osteoprogenitor cells

To stimulate proliferation in bone and to provide an environment to support transplanted cells, we adapted a method of marrow ablation of femur on mature nonsynge-

neic rats as previously described.[26,27] Briefly, a hole 2 mm in diameter was drilled through the growth plate into the marrow channel, and the femur marrow was removed with a polyethylene cannula inserted through the hole and attached to a high-power suction apparatus. Femur marrow was surgically ablated in one leg of nonsyngeniec mature rats. Sixteen hours after surgery, either media or virus-infected metaphyseal or diaphyseal cells preselected in G418 (1×10^6 cells) were injected directly into the ablated marrow channel. Rats were killed 5–6 days later, a time period equivalent to the phase of active bone formation/proliferation that follows ablation. This time point allows identification of the cellular graft and resorption of nonviable cellular material.[26,27] After sacrifice, the presence and localization of the transplanted cells were demonstrated in the primary hosts using three independent approaches: detection of provirus by PCR-Southern blot, ability to recover (viable) transplanted cells from metaphyseal and diaphyseal bone by in vitro clonal selection in G418, and detection of expression of *lacZ* by β-galactosidase histochemistry of bone sections. Femurs were either prepared for histochemistry, or metaphyseal and diaphyseal cells were isolated, selected in G418, and stained for β-galactosidase to determine colony counts or analyzed by Southern analysis to determine the integration pattern. For cell isolation assays, cells from two or three animals were pooled and cultured as described. Metaphysis and diaphysis of both femurs and kidney and spleen of primary hosts were evaluated by PCR-Southern analysis for the presence of the *neo* gene.

PCR analysis

Total cellular DNA was isolated from progenitors using methods that allow for the extraction of nucleic acids from fewer than 1000 cells.[28] Genomic DNA from tissues was isolated using the QIAamp tissue kit (QIAGEN Inc., Chatsworth, CA, U.S.A.) as recommended by the manufacturers. Amplification of genomic DNA was performed as previously described.[21] For amplification of genomic DNA, a 792 bp region of the *neo* gene was amplified using a 27 bp 5′ primer: CAA GAT GGA TTG CAC GCA GGT TCT CCG and ending with the 27 bp 3′ primer: CCA GAG TCC CGC TCA GAA GAA CTC GTC. For amplification of total cellular RNA, an 862 bp region of the *lacZ* gene was amplified using a 27 bp 5′ primer: CAG ATA ACT GCC GTC ACT CCA ACG CAG and a 27 bp 3′ primer: CAG CTT GGG ATC TCT ATA ATC TCG CGC. Each sample was amplified for 30 cycles, at 94°C for 60 s to allow for denaturation, 60°C for 2 minutes to allow for primer annealing, and 72°C for 90 s to allow for primer extension. Twenty microliters of reaction mixture was then electrophoresed on a 2% agarose gel and transferred to a nylon filter (Gene Screen plus, New England Nuclear Corp.). The filter was then hybridized using a 3.7 kb *lacZ* gene fragment or a 0.8 kb *neo* fragment randomly labeled (Boehringer Mannheim) with [^{32}P]dCTP. Hybridization was performed at 42°C for 48 h. Filters were washed with 0.1% sodium dodecyl sulfate (SDS), 0.1× SSC (0.15 M sodium chloride, 0.015 M sodium citrate) at 55°C, and exposed to Kodak XAR film at −80°C. Polyadenylated RNA from tissues was isolated using an RNA isolation kit (QIAGEN Inc.) exactly as described by the manufacturer.

Nucleic acid analysis/Southern blot integration fragment analysis

Total cellular DNA was isolated from cells, using standard phenol chloroform extractions as previously described[21] and digested with a 3- to 5-fold excess of *Dra*I restriction endonuclease (Boehringer Mannheim), using conditions as specified by the manufacturer. DNA from fibroblast cells containing a single copy of the vM5*neolacZ* provirus were used as positive controls. DNA fragments were size separated on a Hoefer SE600 vertical gel apparatus (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.) using a 1.5 mm wide, 1% agarose gel at 22 V for 16-18 h. Gels were prepared for DNA transfer as previously described.[20] Prehybridization of filters was performed in 6× SSC, 0.05× Denhardt's, 0.5% SDS, and 100 mg/ml salmon sperm DNA (Boehringer Mannheim) for 2 h at 68°C. All probes were labeled to a specific activity of 0.5-3.0 × 10^9 dpm/mg with a Prime-It-II random prime labeling kit (Stratagene, La Jolla, CA, U.S.A.) as recommended by the manufacturer using [α-^{32}P]dCTP (Amersham, Arlington Heights, IL, U.S.A.). Hybridization was carried out for 16–18 h at 68°C with 10^7 dpm/ml of either a labeled 1.1 kb *Bam*HI–*Cla*I *lacZ* fragment or an 800 bp *Pvu*II *neo* fragment in hybridization fluid identical in composition to that used for the prehybridization. Following hybridization, filters were washed sequentially in 0.1× SSC, 0.1% SDS at room temperature for 10 minutes, 55°C for 20 minutes and 60°C for 15 minutes Filters were exposed to X-ray film at −80°C with an intensifying screen for 5–28 days.

RESULTS

Harvest and cultivation of metaphyseal and diaphyseal marrow osteoprogenitors

Metaphyseal and diaphyseal marrow osteoprogenitors were cultured from the distal femur of young rats. Twelve to sixteen hours after the initiation of the cultures, adherent cells derived from the metaphyseal primary spongiosa formed distinct foci or aggregates, containing 20–500 mononuclear cells with fibroblastic morphology (Fig. 2A). The nonadherent cell population was removed by replacing the medium with fresh medium. The adherent cells in these aggregates consisted of less than 1% of the total number of cells plated (data not shown). The primary spongiosa–derived cells proliferated rapidly and reached confluence within 5–7 days (Fig. 2B). In cultures established from the diaphyseal marrow, developing colonies could be recognized at 3–6 days as clusters of elongated fibroblasts. The size of the fibroblast foci increased rapidly so that, by 10 days, most of the colonies were composed of hundreds to thousands of cells (Fig. 2C). To demonstrate that proliferating cells retained differentiated functions, cultures were stained cytochemically for ALP. In confluent metaphyseal

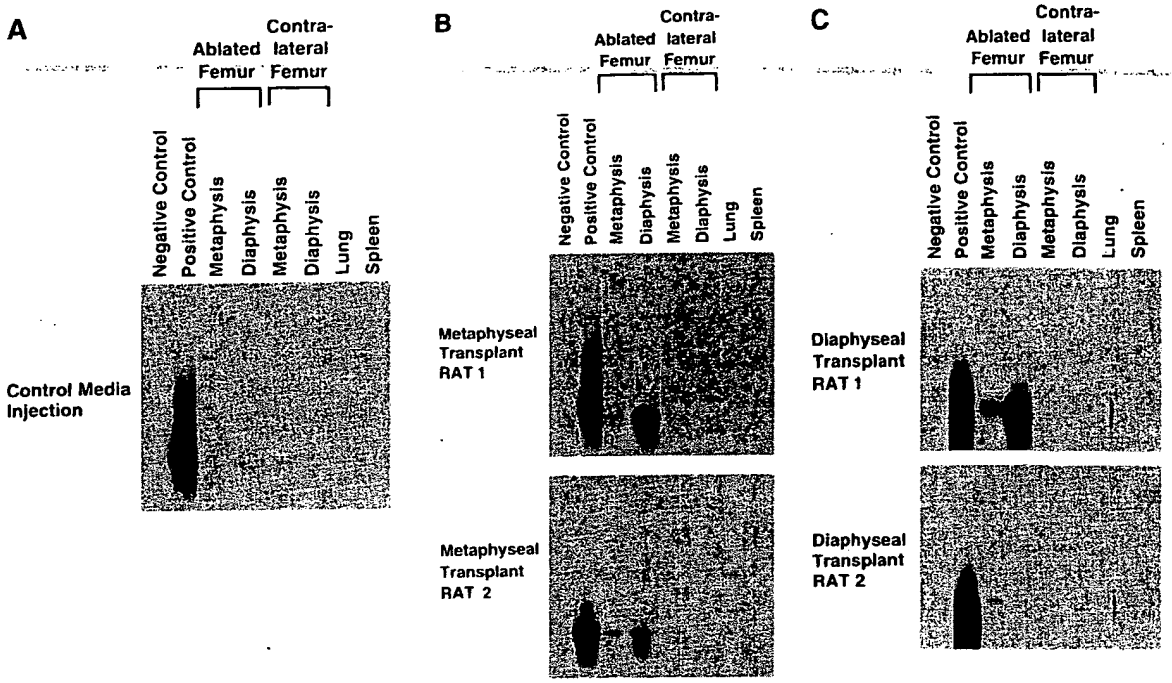

FIG. 4. Detection of proviral sequences in genomic DNA from tissues by PCR plus Southern analysis. Total cellular DNA was isolated from tissues (metaphysis and diaphysis of both femurs, lung and spleen of primary hosts) after transplantation as described and evaluated by PCR for the presence of the *neo* gene. Following amplification, the reaction mixture was then electrophoresed on a 2% agarose gel and transferred to a nylon filter. The filter was then hybridized using an 0.8 kb *neo* fragment randomly labeled with [^{32}P]dCTP. (A) Control media injection, (B) metaphyseal transplant results from two different rats, and (C) diaphyseal transplant results from two different rats are shown. DNA from fibroblast cells containing a single copy of the vM5*neolacZ* provirus wase used as positive control and DNA from uninfected metaphyseal cells was used as negative control.

TABLE 1. DETECTION OF PROVIRUS IN DIFFERENT TISSUES 5–6 DAYS AFTER TRANSPLANTATION

	Injected femur		*Contralateral femur*		*Other tissues*	
Animal #	*Metaphysis*	*Diaphysis*	*Metaphysis*	*Diaphysis*	*Spleen*	*Lung*
Metaphyseal transplant						
1	+	+	–	–	–	–
2	+	+	–	–	–	–
Diaphyseal transplant						
1	+	+	–	–	–	–
2	+	–	–	–	–	–
Control media injection						
1	–	–	–	–	–	–
2	–	–	–	–	–	–

and diaphyseal marrow cell cultures, more than 85% of the cells were cytochemically positive for ALP, and similar ALP activity persisted in passaged cultures (data not shown).[15,18]

Transduction with retrovirus and selection of transduced osteoprogenitor cells with G418

To assess proviral expression in transduced cells, cultures were selected with the neomycin analog, G418. Selected G418-resistant colonies were stained with the chromogenic substrate, X-gal. In control experiments, nontransduced osteoprogenitor cells were uniformly found to be sensitive to 5–7 days of exposure to 1 mg/ml G418. All G418-selected colonies of metaphyseal and marrow-derived osteoprogenitor cells stained positive with X-gal (Figs. 3A and 3B). Cultures of selected cells exhibited morphology similar to that of nonselected cells by phase contrast microscopy. G418-selected cells also retained differentiated function and stained positive for ALP (data not shown).

TABLE 2. G418-RESISTANT COLONIES RECOVERED FROM THE DIAPHYSIS AND METAPHYSIS OF TRANSPLANTED RATS

Experiment # (n = 3)	Metaphyseal transplant		Diaphyseal transplant		Control media injection	
	Metaphysis	Diaphysis	Metaphysis	Diaphysis	Metaphysis	Diaphysis
1	225	0	0	0	0	0
2	368	0	9	2	0	0

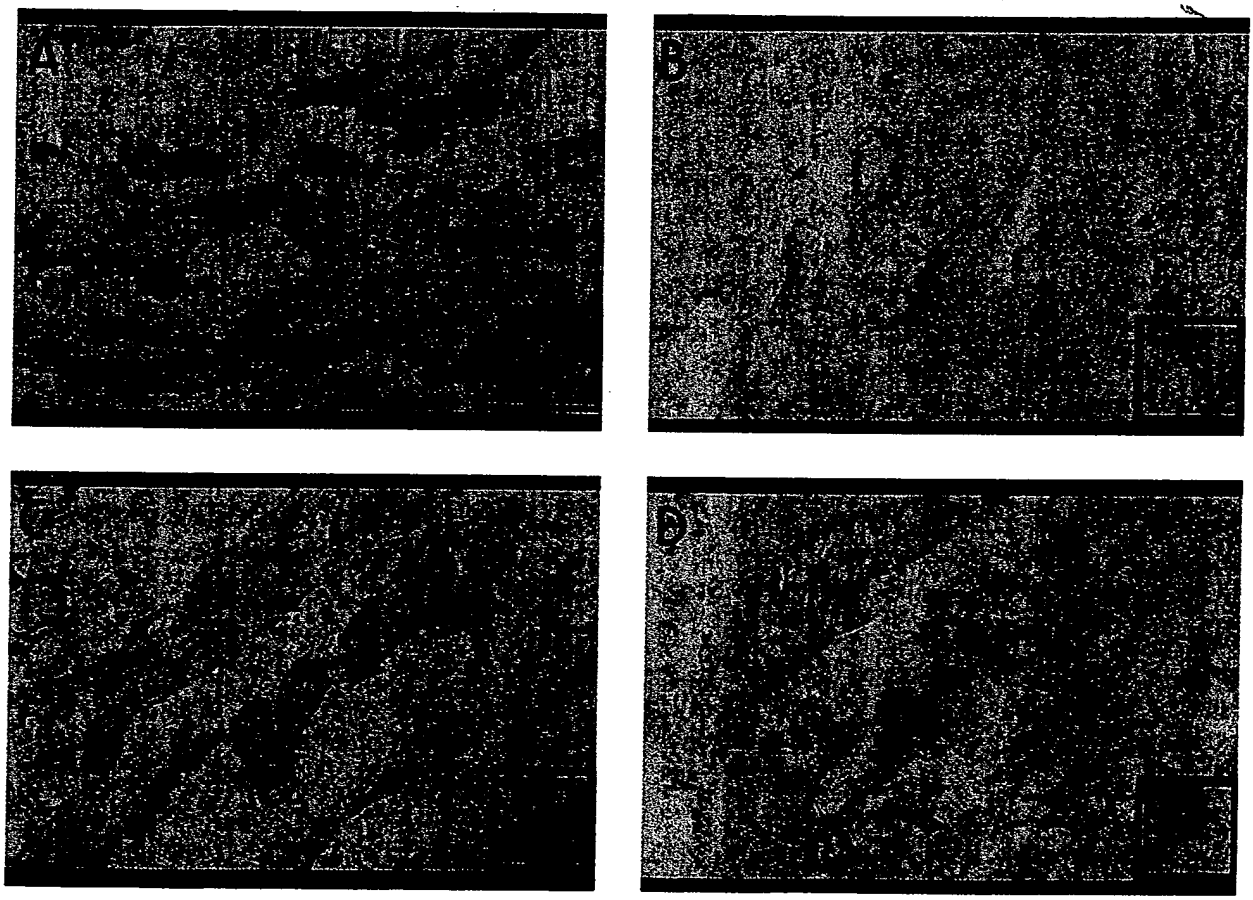

FIG. 5. Photomicrographs of frozen, calcified sections of distal femur to show location of β-galactosidase positive cells in metaphyseal area, 6 days after transplantation of retrovirally infected metaphyseal cells into ablated marrow cavity. β-galactosidase positive cells stain turquoise (arrow). (A) β-galactosidase positive osteoblast and osteoprogenitor cells on trabecular bone surface. (B) β-galactosidase positive osteoblast on trabecular bone surface. (C) β-galactosidase positive cells exhibited terminal osteoblast differentiation as osteocytes within bone. (D) Rarely, β-galactosidase positive cells were present as isolated chondrocytes in the healing cartilage plate.

Transplantation, detection, and recovery of bone osteoprogenitor cells

Transduced metaphyseal or diaphyseal osteoprogenitor cells preselected in G418 or media control were injected into the marrow channel of the ablated femur of nonsyngenic mature rats as described in the Materials and Methods section. To demonstrate the presence of provirus in the primary hosts, DNA was prepared from the metaphysis and diaphysis of both femurs and lung and spleen and analyzed first by PCR and then by Southern blot of the PCR product (Fig. 4 and Table 1). The provirus was detected in DNA from the injected femur in 100% of both metaphyseal and diaphyseal samples, following metaphyseal cell transplants, and in 100% metaphysis and 50% diaphysis, following diaphyseal cell transplants. Provirus was not detected in the contralateral femur or any other tissues examined (Figs. 4B and 4C and Table 1). In addition, the provirus was not detected in any rat tissue when media was injected as a control (Fig. 4A and Table 1).

High numbers of G418-resistant colonies were recovered following metaphyseal transplants (225 and 368 in two independent experiments) and were detected in 100% of

metaphyseal, but in none of the diaphyseal, samples. The recovered G418-resistant colonies represent less than 0.1% of selected cells (data not shown). The number of G418-resistant colonies recovered from the diaphyseal transplants was over 60-fold less compared with metaphyseal cells. Only 0–9 or 0–2 colonies, respectively, were detected in the metaphyseal and diaphyseal specimens. These results are summarized in Table 2. All G418-resistant colonies recovered following metaphyseal and diaphyseal transplants, retained differentiated function, and stained positive for ALP (data not shown).

Following metaphyseal cell transplants, β-galactosidase positive cells were confined to a relatively small fraction of total trabecular bone (Fig. 5). The identity of the cells was defined traditionally by their location and proximity to bone. β-galactosidase positive cells with rounded or columnar morphology, in direct contact with the trabecular bone surfaces, were identified as osteoblasts (Figs. 5A and 5B). Elongated cells with elongated nuclei that were immediately adjacent to osteoblasts and β-galactosidase positive, but did not contact the bone matrix, were classified as putative osteoprogenitor cells (Fig. 5A). β-galactosidase positive cells within lacunae in bone were identified as osteocytes (Fig. 5C). Rarely, positive staining cells were found as chondrocytes within the healing cartilaginous growth plate (Fig. 5D). There were no β-galactosidase positive cells within the bone marrow itself. Histochemical staining of diaphyseal marrow was not done, because few cells were recovered from this region (Table 2). No X-gal positive cells were detected on multiple sequential sections from control animals.

Southern blot integration fragment analysis/cell lineage analysis

Since G418-resistant colonies were readily obtained in the metaphysis following metaphyseal transplant, we assessed whether the G418-resistant colonies were derived from the same progenitor cell(s). This assessment was determined by the ability to analyze the random integrations of proviral DNA in chromosomal DNA from metaphyseal cells recovered after transplantation into ablated femurs. High molecular weight genomic DNA was digested with a restriction endonuclease which cleaves both proviral and flanking chromosomal DNA followed by Southern blotting of the digested DNA fragments. Hybridization of the blot with the *lacZ* probe allowed identification of the 5′ region of the provirus and the flanking chromosomal DNA 5′ to the provirus, while hybridization of the filter with the *neo* probe allowed identification of the 3′ region of the provirus and chromosomal DNA flanking the 3′ junction. In the first experiment, the integration pattern of multiple individual colonies from a pool of three animals (Fig. 6A, lanes 1–3) was compared with the integration pattern of metaphyseal cells obtained prior to the transplant (Fig. 6A, lane 5). A commonly sized hybridizing band was detected in cells prior to, and subsequent to, the transplant. Matching hybridizing bands from both the 5′ and 3′ junctions in the metaphyseal cells prior to and following transplantation strongly infers the transduction as well as the in vitro and in vivo proliferation

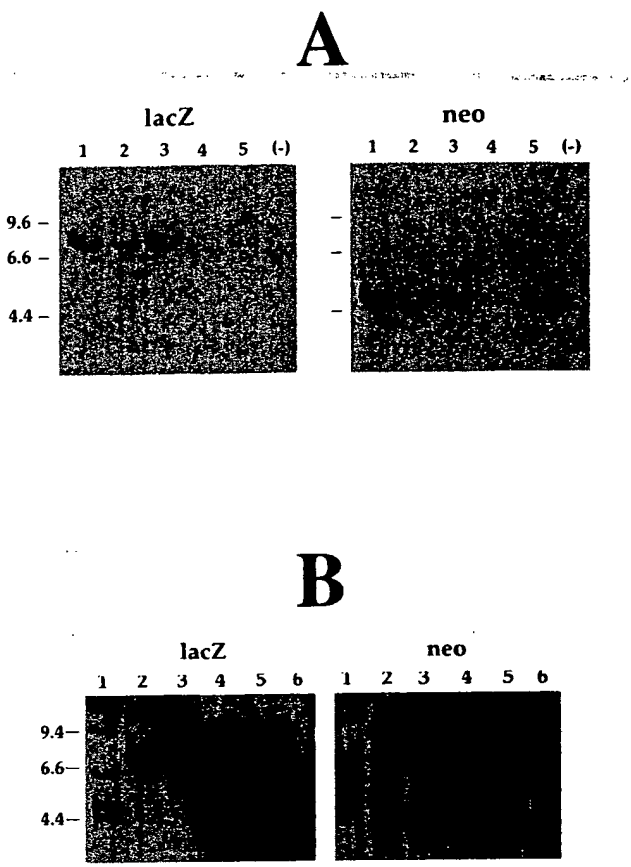

FIG. 6. Analysis of integration fragments in G418-resistant metaphyseal cells. DNA was prepared from metaphyseal cells after in vitro transduction and following transplantation into recipient rats. DNA was digested with *Dra*I restriction endonuclease which cleaves the proviral DNA once and flanking 5′ and 3′ genomic DNA at unique sites to allow the identification of proviral integrations into DNA by Southern blot. The filters were first hybridized with the *lacZ* probe (5′ integration fragment) and then stripped and rehybridized with the *neo* probe (3′ integration fragment). Details are provided in the Materials and Methods section. (A) Lanes 1–3 represent individual G418-resistant clones recovered from transplanted animals; lane 5 represents the parent pool of metaphyseal cells; lane 4 did not contain DNA; lane 6 (-) represents the negative control, uninfected metaphyseal cells. (B) Lanes 2–5 represent pools of G418-resistant clones recovered from transplanted animals; lane 1 represents the parent pool of metaphyseal cells; lane 6 represents the negative control, uninfected metaphyseal cells. DNA from fibroblast cells containing a single copy of the vM5*neolacZ* provirus was used as positive control and yielded expected results (data not shown).

of a common osteoprogenitor cell. In a second experiment (Fig. 6B), five hybridizing bands, representing five distinct proviral integrations, were observed in genomic DNA isolated from cells prior to transplantation (Fig. 6B, lane 1). However, following transplantation, a single integrant was observed (Fig. 6B, lanes 2–5) in pooled colonies. The integrant observed in the transplanted cells differed from any of the integrants

obtained from genomic DNA isolated from cells prior to transplantation.

DISCUSSION

We transplanted metaphyseal osteoprogenitors (the source of trabecular osteoblasts) and diaphyseal marrow osteoprogenitors (the source of endosteal osteoblasts), isolated from bones of young rats, into marrow ablated-femur bones of nonsyngeneic mature rats to investigate their fate during the phase of rapid bone formation, which occurs immediately after surgical marrow ablation.[26,27] To distinguish and facilitate identification and recovery of transplanted cells, the transplanted cells were infected with vM5*neolacZ* retrovirus encoding the neomycin resistance gene *neo* and the histochemical reporter gene *lacZ*.[19,20,29–31] A similar somatic gene transfer approach has been used in research on stem cells of the hematopoietic system where the production of vast numbers of hematopoietic cells is hypothesized to be maintained by a small population of reconstituting stem cells.[19–21,31–36] The methodology generally involves bone marrow harvest, in vitro transduction, and transplantation of the infected cells into irradiation-conditioned recipients. The proliferative properties of the stem and progenitor cells are inferred by Southern blot detection of the proviral integrations in mature hematopoietic lineages. Our results demonstrate that 5–6 days following transplantation, the provirus was detected only in the injected femur (metaphyseal and diaphyseal region) and not in the contralateral femur or in any other tissue examined. The absence of the provirus in organs other than the injected femur indicate a limited circulation of these bone osteoprogenitor cells or a failure to engraft/survive in these sites.

Efforts to recover G418-resistant cells from the femoral metaphysis and diaphysis showed high numbers of G418-resistant colonies in metaphyseal sites, but none in diaphyseal samples, following metaphyseal transplants. This suggests that metaphyseal osteoprogenitor cells, when transplanted into bone, engraft into trabecular bone of the metaphysis. We have previously characterized these cells in vitro. We have shown that these cells aggregate within hours after isolation and express osteopontin, collagen I, and ALP mRNA. After 4 days in culture, transcripts for the PTH receptor and ALP increase, and spontaneous mineralization occurs 2-3 days later, without supplementation of the media.[15,18,37] Proliferation of serum-starved cells above baseline is significantly stimulated by the addition of serum, but not by isolated growth factors, dexamethasone, or PTH.[15,18,37] In diffusion chambers implanted into the peritoneal cavity of athymic mice, these metaphyseal cells have the ability to give rise to bone tissue (unpublished data, Santerre and Onyia, 1997).

Few or no G418-resistant colonies were obtained from diaphyseal marrow transplants, although G418-resistant colonies of diaphyseal origin were detected in both metaphyseal and diaphyseal samples. The significance of this difference in proliferation between the metaphyseal and diaphyseal osteoprogenitor cells in our studies and those reported by others is unclear, though it may be related to the transplantation procedure and the time of analysis. Another possibility is that there may be differences in the in vivo growth rates of the two populations. In vivo studies by us and others have shown that the metaphysis contains many more cells that are rapidly dividing than does the diaphysis.[13,14,16] Under steady-state conditions in vivo, the diaphyseal cells have a slow rate of mitosis, and more than 90% remain arrested in the Go-period of the cell cycle, but are stimulated to proliferate and differentiate when transplanted into nutrient-rich media in vitro.[38–42] In vitro, as determined by both cell counting and [^{3}H]thymidine incorporation, we have not found significant differences in proliferation between the metaphyseal and diaphyseal cells that could account for the different outcome in vivo (unpublished results, J.E. Onyia).

Pereira et al.[43] transplanted diaphyseal marrow stromal cells from transgenic mice expressing a human mini-gene for collagen I, as a marker to follow the fate of donor cells. After injection of tagged cells, few donor cells were detected in bone or marrow of nonirradiated recipient mice, while donor marrow stromal cells were widely disseminated and populated bone, marrow, cartilage, lung, and spleen in irradiation-conditioned mice. In contrast, in our study, using a different subset of bone cells and a different bone preparatory regimen (ablation), a highly efficient engraftment in a localized bone region was observed. It will be important in our future studies to evaluate the proliferation of metaphyseal and diaphyseal cells in bone at several points in time following transplantation, because one population of cells may have short-term proliferating potential while the other population has long-term proliferation potential.

Histologic sections showed that the β-galactosidase metaphyseal osteoprogenitors were able to compete successfully with endogenous bone cells and complete their differentiation within the osteoblast lineage as osteoprogenitors, osteoblasts, and osteocytes within metaphyseal bone. Although these cells vigorously proliferated in vitro, they were not located within the marrow itself as clusters of cells, but as the occasional osteoprogenitor within the primary spongiosa. The observation that β-galactosidase positive chondrocytes were found, albeit rarely, within the growth plate, suggests that these metaphyseal cell transplants contained cell(s) that were at least bipotential and capable of differentiating into osteoblast or chondrocyte lineages, as suggested by previously published in vitro studies.[22,43–47]

We found some discrepancy in PCR analysis and the results of cell isolation with the metaphyseal cell transplants. While G418-resistant cells were only isolated from the metaphysis, provirus was detected in both metaphyseal and diaphyseal samples. The difference may be attributed to the high sensitivity of the PCR technique in detecting the presence of very rare contaminating metaphyseal cells containing the provirus in these specimens (limits of detection 1/100,000 cells; unpublished results, D.W. Clapp). Alternatively, it may be that the transplanted metaphyseal cells are not proliferating in the diaphyseal microenvironment at the time of sampling or that diaphyseal marrow environment

cannot support the growth/survival of metaphyseal osteoprogenitors.

Analysis of proviral insertion sites in genomic DNA of recovered metaphyseal cells was determined to evaluate the cell fates of individual transduced cells. In the first experiment (Fig. 6A), the integrant detected in DNA recovered from the femurs of the transplanted rats was the same as that observed in the metaphyseal cells prior to transplantation, indicating the ability of a single transduced cell to survive in vivo as well as in vitro. The integration pattern observed in the second experiment was more complex. While data in Fig. 6A indicate that a transduced cell can proliferate in vitro and survive in vivo, the relative in vivo survival of multiple cells could not be determined. In an independent experiment shown in Fig. 6B, five distinct integrants were observed in the input metaphyseal cells, though not in DNA from cells recovered from the transplanted rats. The detection of the five integrants in vitro, though not in vivo, suggests that cells containing these integrants failed to engraft and survive in vivo, or that the cells differentiated in vitro prior to transplantation. One integrant was detected in DNA from the reconstituted rats, though not in the input metaphyseal cells. It is possible that there was a rearrangement of one of the integrants detected in vitro, resulting in the presence of a uniquely sized integrant. Alternatively, the data are also consistent with the proliferation of a cell in vivo that was not proliferating sufficiently in vitro to be detected. Such clonal fluctuation has been noted frequently following transplantation of retroviral-marked hematopoietic stem and progenitor cells.[20,21,25,31–35] It will be interesting in future experiments to evaluate the clonal proliferation of transduced metaphyseal cells as a function of time. Such an approach has been used to characterize the proliferation kinetics of circulating and bone marrow hematopoietic cells in murine models.[20,21,25,31–35]

The ability to target bone for gene therapy will depend on the presence of primitive osteoprogenitors which possess a high proliferative capacity in vitro and in vivo. Further studies will be required to assess the long-term function of the transplanted cells, as well as the persistence of gene expression from the integrated provirus. This is the first demonstration of successful and selective transplanting of clonal osteoprogenitors to their site of origin in bone. Also these initial studies establish the feasibility of ex vivo gene delivery to bone. This suggests that, with appropriate engineering, it may be possible to use these cells to target genes to bone for therapeutic use in skeletal and hematopoietic disease.

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REFERENCES

1. Caplan AI 1991 Mesenchymal stem cells. J Orthop Res 9: 641-650.
2. Caplan AI 1987 Bone development and repair. BioEssays 6: 171-175.
3. Bab I, Howlett CR, Ashton BA, Owen ME 1984 Ultrastructure of bone and cartilage formed in vitro in diffusion chambers. Clin Orthop 187:243-254.
4. Ohgushi H, Goldberg VM, Caplan AI 1989 Heterotopic osteogenesis in porous ceramics induced by marrow cells. J Orthop Res 7:568-578.
5. Ohgushi H, Goldberg VM, Caplan AI 1989 Repair of segmental long bone defect by composite graft of marrow cells and porous calcium phosphate ceramic. Acta Orthop Scand 60:334-339.
6. Nakahara H, Bruder SP, Goldberg VM, Caplan AI 1990 In vivo osteochondrogenic potential of cultured cells derived from the periosteum. Clin Orthop Rel Res 259:223-232.
7. Lucas PA, Syftestad GT, Caplan AI 1988 A water-soluble fraction from adult bone stimulates the differentiation of cartilage in explants of embryonic muscle. Differentiation 37: 47-52.
8. Nathanson MA, Hay ED 1980 Analysis of cartilage differentiation from skeletal muscle grown in bone matrix I: Ultrastructural aspects. Dev Biol 78:301-331.
9. Owen M 1985 Lineage of osteogenic cells and their relationship to the stromal system. In: Peck WA (ed.) Bone and Mineral Research, Vol 3. Elsevier, New York, NY, U.S.A., pp. 1-25.
10. Bab I, Passi-Even L, Gazit D, Sekeles E, Ashton BA, Peylan-Ramu N, Ziv I, Ulmansky M 1988 Osteogenesis in in vivo diffusion chamber cultures of human marrow cells. Bone Miner 4:373-386.
11. Herbertson A, Aubin JE 1995 Dexamethasone alters the subpopulation make-up of rat bone marrow stromal cell cultures. J Bone Miner Res 10:285-294.
12. McCulloch CA, Strugurescu M, Hughes F, Melcher AH, Aubin JE 1991 Osteogenic progenitor cells in rat bone marrow stromal populations exhibit self-renewal in culture. Blood 77: 1906-1911.
13. Kimmel DB, Wss J 1980 Bone cell kinetics during longitudinal bone growth in the rat. Calcif Tissue Int 32:123-133.
14. Kember NF 1960 Cell division in endochondral ossification: A study of cell proliferation in rat bones by the method of of triated thymidine autoradiography. J Bone Joint Surg 42: 824-839.
15. Onyia JE, Miller B, Hulman J, Liang J, Galvin R, Frolik C, Chandrasekhar S, Harvey AK, Bidwell J, Herring J, Hock JM 1997 Proliferating cells in the primary spongiosa express osteoblastic phenotype in vitro. Bone 20:93-100.
16. Hock JM, Onyia JE, Miller B, Hulman J, Herring J, Chandrasekhar S, Harvey AK, Gunness M 1994 Anabolic PTH targets proliferating cells of the primary spongiosa in young rats, and increases the number differentiating into osteoblasts. J Bone Miner Res 9:S412 (abstract).
17. Hock JM, Onyia JE, Bidwell J 1995 Comparisons of in vivo and in vitro models of the response of osteoblasts to hormonal regulation with aging. Calcif Tissue Int 56:S44-S47.
18. Alvarez M, Long H, Onyia J, Hock J, Xu W, Bidwell J 1997 Rat osteoblast and osteosarcoma nuclear matrix proteins bind with sequence specificity to the rat type I collagen promoter. Endocrinology 138:482-489.
19. Clapp DW, Freie B, Srour E, Yoder MC, Fortney K, Gerson SL 1995 Myeloproliferative sarcoma virus directed expression of beta-galactosidase following retroviral transduction of murine hematopoietic cells. Exp Hematol 23:630-638.
20. Clapp DW, Freie B, Lee W-H, Zhang Y-Y 1995 Molecular evidence that in situ-transduced fetal liver hematopoietic stem/progenitor cells give rise to medullary hematopoiesis in adult rats. Blood 86:2113-2122.
21. Clapp DW, Dumenco LL, Hatzoglou M, Gerson ST 1991 Fetal

liver hematopoietic stem cells as a target for in utero retroviral gene transfer. Blood **78**:1132-1139.
22. Owen M 1988 Marrow stromal stem cells. J Cell Sci **10**:63-76.
23. Galvin RJS, Cullison JW, Avioli LV, Osdoby PA 1994 Influence of osteoclasts and osteoclast-like cells on osteoblast alkaline phosphatase activity and collagen synthesis. J Bone Miner Res **9**:1167-1177.
24. Lin W-C, Pretlow TP, Pretlow TG II, Culp LA 1990 Bacterial lacZ gene as a highly sensitive marker to detect micrometastasis formation during tumor progression. Cancer Res **50**: 2808-2817.
25. Sanes JR, Rubenstein JLR, Nicolas J-F 1986 Use of a recombinant retrovirus to study post-implantation cell lineage in mouse embryos. EMBO J **5**:3133-3142.
26. Suva LJ, Seedor JG, Endo N, Quartuccio HA, Thompson DD, Bab I, Rodan GA 1993 Pattern of gene expression following rat tibial marrow ablation. J Bone Miner Res **8**:379-388.
27. Bab I, Gazit D, Massarawa A, Sela J 1985 Removal of tibial marrow induces increased formation of bone and cartilage in rat mandibular condyle. Calcif Tissue Int **37**:551-555.
28. Higuchi R 1989 Rapid efficient DNA extraction for PCR from cells or blood. Amplifications **1**:3.
29. Eglitis MA, Anderson WF 1988 Retroviral vectors for introduction of genes into mammalian cells. BioTechniques **6**: 608-613.
30. O'Malley BWJr, Adams RM, Sikes ML, Sawada T, Ledley FD 1993 Retrovirus-mediated gene transfer into canine thyroid using an ex vivo strategy. Hum Gene Ther **4**:171-178.
31. Keller GL, Snodgrass RJ 1990 Life span of multipotential hematopoietic stem cells in vivo. Exp Med **171**:1407-1418.
32. Williams DA, Lemischka IR, Nathan DG, Mulligan RC 1984 Introduction of new genetic material into pluripotent haematopoietic stem cells of the mouse. Nature **310**:476-479.
33. Keller G, Paige C, Gilboa E, Wagner EF 1985 Expression of a foreign gene in myeloid and lymphoid cells derived from multipotent haematopoietic precusors. Nature **318**:149-154.
34. Jordan CT, Lemischka IR 1990 Clonal and systemic analysis of long-term hematopoiesis in the mouse. Genes Dev **4**:220-232.
35. Lemischka IR, Raulet DH, Mulligan RC 1986 Developmental potential and dynamic behavior of hematopoietic stem cells. Cell **45**:917-927.
36. Brecher G, Bookstein N, Redfearn W, Necas E, Pallavicini MG, Cronkite EP 1993 Self-renewal of the long-term repopulating stem cell. Proc Natl Acad Sci USA **90**:6028-6031.
37. Long H, Onyia JE, Hulman J, Bidwell J, Hock JM 1995 Molecular characterization of primary spongiosa cells from distal femurs of young rats. J Bone Miner Res **10**:M237 (abstract).
38. Friedenstein AJ, Chailakhjan RK, Lalykina KS 1970 The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet **3**:393-403.
39. Epikhina S, Iu Latsinik NV 1976 Proliferative activity of stromal cells-bone marrow precursors possessing clonogenic properties. Biull Eksp Biol Med (USSR) **81**:55-57.
40. Kaneko S, Motomura S, Ibayashi H 1982 Differentiation of human bone marrow-derived fibroblastoid colony forming cells (CFU-F) and their roles in haemopoiesis in vitro. Br J Haematol **51**:217-225.
41. Keilis-Borok IV, Latsinik NV, Epikhina SI, Fridenshtein AI 1971 Dynamics of formation of fibroblast colonies in monolayer cultures of bone marrow according to 3H-thymidine incorporation data. Tsitologiia (USSR) **13**:1402-1409.
42. Friedenstein AJ, Latzinik NV, Gorskaya EA, Luria EA, Moskvina IL 1992 Bone marrow stromal colony formation requires stimulation by haemopoietic cells. Bone Miner **18**:199-213.
43. Pereira RF, Halford KW, O'hara MD, Leeper DB, Sokolov BP, Pollard MD, Bagasra O, Prockop DJ 1995 Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. Proc Natl Acad Sci USA **92**:4857-4861.
44. Friedenstein AJ, Chailakhyan RK, Gerasimov UV 1987 Bone marrow osteogenic stem cells: In vitro cultivation and transplantation in diffusion chambers. Cell Tissue Kinet **20**:263-272.
45. Friedenstein AJ, Latzinik NW, Grosheva AG, Gorskaya UF 1982 Marrow microenvironment transfer by heterotopic transplantation of freshly isolated and cultured cells in porous sponges. Exp Hematol **10**:217-227.
46. Leboy PS, Beresford JN, Devlin C, Owen ME 1991 Dexamethasone induction of osteoblast mRNAs in rat marrow stromal cell cultures. J Cell Physiol **146**:370-378.
47. Mardona HJ, Bee J, Mark KVD, Owen ME 1987 Development of osteogenic tissue in diffusion chambers from early precusor cells in bone marrow of adult rats. Cell Tissue Res **250**:157-165.
48. Stocking C, Koller R, Bergholz U, Ostertag W 1985 Long terminal repeat sequences impart hematopoietic transformation properties to the myeloproliferative sarcoma virus. Proc Natl Acad Sci USA **82**:5746-5750

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